AMMONIA EXCRETION IN THE ATLANTIC HAGFISH, (*Myxine glutinosa*)

A Thesis
by
JUSTIN MATTHEW ARNOLD

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2013
Department of Biology

AMMONIA EXCRETION IN THE ATLANTIC HAGFISH,
(*Myxine glutinosa*)

A Thesis
by
JUSTIN MATTHEW ARNOLD
December 2013

APPROVED BY:

Susan L. Edwards, Ph.D.
Chairperson, Thesis Committee

Ted Zerucha, Ph.D.
Member, Thesis Committee

Patrick J. Walsh, Ph.D.
Member, Thesis Committee

Susan L. Edwards, Ph.D.
Chairperson, Department of Biology

Edelma D. Huntley, Ph.D.
Dean, Cratis Williams Graduate School

Abstract

AMMONIA EXCRETION IN THE ATLANTIC HAGFISH,
(*Myxine glutinosa*)

Justin Matthew Arnold
B.S., Appalachian State University
M.S., Appalachian State University

Chairperson: Susan L. Edwards, Ph.D.

The hagfishes possess the longest known evolutionary history of the extant craniates and likely offer exclusive insight into vertebrate origins. The Atlantic hagfish is physiologically adapted to spend much of its life burrowed in ocean-floor substrate and marine carcasses, where unfavorable conditions for ammonia excretion are likely encountered. Plasma ammonia concentrations were experimentally elevated by injection of NH_4Cl. Ammonia excretion rates and plasma ammonia concentrations were measured. Expression of Rhcg and Rhbg mRNA was quantified using quantitative RT-PCR. We present evidence that suggests Atlantic hagfish are capable of reducing experimentally elevated plasma ammonia concentrations and eventually eliminating the plasma ammonia load within 12 hours. Following the injection of ammonia elevated plasma ammonia concentrations paralleled elevated ammonia excretion rates and coincided with the initial upregulation of Rhcg and Rhbg in the gill and Rhcg in the skin suggesting that the transcriptional regulation of Rh glycoproteins may respond, in part, to elevated plasma ammonia.

Acknowledgments

Very few things worth accomplishing are completed independently of the direction, support and encouragement of others. This project certainly was no exception. I wish to acknowledge and offer my gratitude to Dr. Susan Edwards for her patient advising and for allowing me to pursue this research with little prior experinece in molecular biology or protein biochemistry. I would like to thank my thesis committee members: Dr. Ted Zerucha for his time and input and especially Dr. Patrick Walsh for the cloning of Rhcg and Rhbg in the Atlantic hagfish and his willingness to travel for the defense of this thesis. I would have been even more lost in my first semester of graduate studies without the willingness and aptitude of Brian Mikeworth in teaching me the basics of molecular cloning and western blotting, while writing his own thesis. Brian, only now do I realize what a sacrifice you were making. Many thanks are due to Margo Pray and Ryland Bradley for their invaluable assistance and lively spirits during otherwise tedious work. I am indebted to Dr. J.B. Claiborne for his technical assistance, without which I would likely still be calculating net ammonia flux. Without Dr. Dave Evans, I would have learned much less about the history of comparative fish physiology and would not have had the hooks on which to hang much of what I have learned since my summer at MDIBL. Thanks to Dr. Sue Bauldry for directing the production of the homologous hagfish Rhcg antibody through an immunology laboratory course. This project and my involvement in it would not have been possible without generous funding from the NSF (grant # 1OS 112136 awarded to SLE).

Dedication

To my loving wife, Joanna, who graciously and patiently supported me through the adventure of graduate studies, while embarking on her own voyage into motherhood. Your tremendous investment in our family cannot be fully appreciated through words alone.

Table of Contents

List of Tables

List of Figures

Introduction

By the late 1930s, Homer Smith (1929) had discovered that fish excrete ammonia across the gill epithelium and August Krogh was investigating the ionoregulatory relationship between sodium (Na^+) uptake and the excretion of ammonia in freshwater fishes (Krogh, 1939). In ostensibly unrelated research, Rhesus (Rh) proteins were determined to be an antigen important to blood immunocompatibility (Levine and Stetson, 1939). Decades later, the discovery of Rh glycoproteins (Marini et al., 1997) and the proposition of an evolutionarily conserved functional role in ammonia transport (Huang and Peng, 2005) emerged. For those interested in understanding the mechanisms of ammonia transport and excretion, research has continued for many decades and teleost fish have remained among the choice model organisms for its study (Evans, 2011; Ip and Chew, 2010). Recently, a resurgence of scientific investigation has occurred; aimed at revealing the role of Rh glycoproteins in ammonia excretion within a broad range of organisms through structural, functional and histological analyses (Weihrauch et al., 2009; Wright and Wood, 2009).

Ammonia excretion

Ammonia is the nitrogenous waste product of amino acid catabolism, which, due to its toxicity, must be excreted directly or converted into less toxic metabolites, such as urea or uric acid (Ip et al., 2001; Wright, 1995). Note that ammonia can exist in a non-ionic, gaseous form (NH_3) or ionic form (NH_4^+) and will be referred to simply as 'ammonia' when the form is not specified. When the chemical form needs to be specified, gaseous ammonia will be

referred to as NH_3 or ammonia gas and ionic ammonia as NH_4^+ or ammonium ion. The term 'total ammonia' will be used to refer to NH_3 and NH_4^+, collectively. Disruption of an organism's ability to excrete or metabolize ammonia leads to a state of hyperammonemia (an excess of ammonia in the blood) that in vertebrates results in nerve and tissue damage (Cooper and Plum, 1987; Walsh et al., 2007) and in mammals leads to impaired cognitive function (hepatic encephalopathy) (Monfort et al., 2009). The direct excretion of ammonia is metabolically inexpensive; however, it requires copious amounts of water to adequately dilute the ammonia to below toxic concentrations (Ip and Chew, 2010). Thus, with a few exceptions involving the volatilization of ammonia in terrestrial snails (Speeg and Campbell, 1968), terrestrial crabs (Greenaway and Nakamura, 1991), and terrestrial isopods (Wieser et al., 1969), only aquatic organisms are capable of excreting ammonia directly (Wright, 1995). With this in mind, it is not surprising that the vast majority of aquatic organisms are ammonotelic, excreting their nitrogenous waste predominantly as ammonia (Walsh, 1998). Despite relatively greater metabolic costs, some aquatic organisms such as elasmobranchs, the Lake Magadi tilapia (*Alcolapia grahami*), and the gulf toadfish (*Opsanus beta*), metabolize ammonia via the ornithine urea cycle and are (at least facultatively) ureotelic, excreting nitrogenous waste predominantly as urea (Walsh et al., 2001). In contrast, terrestrial organisms must conserve water to varying degrees and consequently must invest energy in the metabolism of ammonia into urea or uric acid (Reviewed in Wright, 1995).

In most fishes, nitrogenous waste is excreted primarily across the branchial (gill) epithelium as ammonia (Evans et al., 2005; Smith, 1929), although a number of studies suggest that cutaneous, intestinal, and renal routes may also play a significant role in ammonia excretion when branchial surface area is reduced or compromised (Reviewed in

Wright and Wood, 2012). Krogh (1939) suggested that the excretion of ammonium ion might be associated with an influx of sodium, especially in freshwater, where the uptake of sodium is an essential part of osmoregulation. Subsequent investigations indicated that Na^+/NH_4^+ exchange does occur in freshwater fish, though likely in conjunction with the diffusion of NH_3 across the branchial epithelium (Maetz and Garcia Romeu, 1964). It was thought that NH_3 could readily diffuse across the lipid bilayers and paracellular junctions of the branchial epitelium and that a favorable diffusion gradient was maintained by the protonation of NH_3 (forming NH_4^+) with H^+ produced by the hydration of CO_2 (Wright et al., 1989). Decades of research evaluating Na^+/NH_4^+ exchange in fish have produced conflicting results and suggest that in many species Na^+/H^+ exchange is predominant (Evans, 2011). A large proportion of ion-transport is accomplished through mitochondrion-rich cells (MRCs), ionocytes, usually located on the interlamellar surfaces of the gill filament (Evans et al., 2005). The MRCs of freshwater fish contain Na^+/K^+-ATPase (NKA) and in saltwater fishes, the $Na^+/K^+/2Cl^-$ co-transporter (NKCC) is also present (Wilkie, 2002). Due to similarities in hydrated ionic radii, NH_4^+ is able to substitute for K^+ as a substrate for NKA (Towle and Holleland, 1987) and NKCC (Good et al., 1984), allowing transport of ammonia across the basolateral (serosal or blood-side) membrane of the MRCs. The proposition of apical (mucosal or water-side) Na^+/NH_4^+ exchange has proved more controversial, in part due to concerns regarding improbable thermodynamics (Evans, 2011; Kumai and Perry, 2011; Tsui et al., 2009). Prior to recent studies, it was thought that the simple diffusion of ammonia gas across the lipid bilayers of the branchial epithelium was the major mechanism of NH_3 transport, occurring without the need for a specialized ammonia gas channel to facilitate diffusion (Evans et al., 2005; Wilkie, 2002). However, epithelial cell membrane

permeability (especially the apical membrane) to NH_3 can be relatively low and may be insufficient to account for observed excretion rates (Kikeri et al., 1989). The investigation of additional means of ammonia transport, including ammonia channels, is thus warranted. Ammonia excretion is likely facilitated by a variety of transcellular and paracellular mechanisms, both active and passive, but the potential involvement of Rh glycoproteins has sparked a reevaluation of the mechanisms of ammonia excretion (Reviewed in Weihrauch et al., 2009; Wright and Wood et al., 2009).

Rh glycoproteins

The relatively recent discovery of Rh glycoproteins as part of a larger superfamily, including the methylammonium permases and ammonium transporters (MEP/Amt/Rh) (Huang and Peng, 2005) provides a viable candidate as a potential ammonia channel and is supported by both functional evidence and sequence homology with the Mep/Amt family of NH_4^+ transporters (Marini et al., 1997; reviewed in McDonald et al., 2012). The Rh protein family includes non-glycosylated proteins (Rh-30 group) involved in blood immunocompatibility and glycosylated (Rh-50 or Rh glycoprotein) proteins, which are implicated in ammonia transport (Cherif-Zahar et al., 2007). The Rh-30 proteins were initially discovered as the pathological agent of erythroblastis fetalis, or hemolytic disease of the newborn (Levine and Stetson, 1939), and have become an important factor in blood typing (Westhoff, 2007). However, the physiological function of the Rh-50 proteins was not known until the characterization of human RhAG expressed in yeast (Marini et al., 2000) and *Xenopus* oocytes (Ripoche et al., 2004) and human RhBG expressed in *Xenopus* oocytes (Ludewig, 2004) indicated the capacity for ammonia transport.

Rh nomenclature allows differentiation of the blood antigens, genes, and the proteins. In humans, the blood antigens or Rh factors are designated as D, C, or E. Capital letters denote the RH genes, with or without italics, including erythroid RHD, RHCE, and RHAG, as well as the non-erythroid homologs, RHBG and RHCG. Proteins are indicated with a lower-case "h" (RhD, RhCE, RhAG, RhBG, and RhCG). Addition of a "G" to a gene or protein name denotes glycosylation of the translated protein, thus glycoproteins (Westhoff, 2007). In non-human organisms, only the "R" is capitalized, i.e. Rhcg (Han et al., 2009).

Early bioinformatics analyses predicted that, in most organisms, Rh family proteins would primarily function as CO_2 channels with only residual ammonia transport functionality left over from the Amt ancestry (Cherif-Zahar et al., 2007). Further predictions estimated the absence of Rh genes and predominance of Amt in bacteria and plants, likely due to the benefits of nitrogen uptake imparted by Amt and inefficiency of Rh in low CO_2 conditions. The negative effects of nitrogen retention caused by the toxicity of the metabolically produced nitrogenous wastes in vertebrates may explain apparent retention of Rh genes, while Amt genes have been lost (Cherif-Zahar et al., 2007). Matassi et al. (1999) presented evidence for multiple gene duplications within the Rh gene family of primates. The estimated occurrence of the first duplication event approximately 250-346 million years ago is thought to have enabled evolutionary divergence between the genes of the Rh-30 and Rh-50 groups. The apparent functional significance of the Rh-50 genes in ammonia transport within a variety of distantly related species is thought to have resulted in a slower rate of evolution and thus higher evolutionary conservation of function relative to the Rh-30 genes, while relaxed selective pressure may have resulted in a new function and the loss of glycosylation in the Rh-30 group (Matassi et al., 1999).

Non-mammalian vertebrates generally express three groups of Rh isoforms: Rhag, Rhbg, and Rhcg (Wright and Wood, 2009). Elasmobranchs and some teleost fishes also express the primitive isoform Rhp2 (Huang and Peng, 2005; Nakada et al., 2010), and the teleost fish studied to date are known to express two variants (Rhcg1 and Rhcg2) of Rhcg isoform (Reviewed in Weihrauch et al., 2009); however, invertebrates express the primitive isoform Rhp1, which has not yet been identified in the vertebrates (Huang and Peng, 2005; Nakada et al., 2010; Weihrauch et al., 2009).

Rh glycoproteins in fish

Evidence supporting the capacity of Rh glycoproteins to transport ammonia and their predicted presence in an evolutionarily diverse array of organisms has paved the way for multiple studies focused on confirming the presence of Rh isoforms and elucidating their role in the regulation of ammonia excretion in various animal models. Fish are an excellent model for such investigations as most species generally excrete nitrogenous waste in the form of ammonia (Ip and Chew, 2010).

Using *in-situ* hybridization and immunolocalization in gill tissues along with functional analysis by heterologous Rh glycoprotein expression in *Xenopus* oocytes, a study by Nakada et al. (2007a) presented evidence suggesting that the pufferfish (*Takifugu rubripes*) utilizes multiple Rh glycoproteins for ammonia excretion. The localization of Rh glycoprotein mRNA and protein expression in the gill lamellae allowed the development of a working model for Rh glycoprotein mediated ammonia excretion in pufferfish (Fig. 1). Rhag was located both apically and basolaterally on pillar cells, facilitating ammonia transport from the blood to the basal lamina (Fig. 1B). Basolateral expression of Rhbg and apical expression of Rhcg2 is predicted to facilitate transcellular movement of ammonia across the

pavement cell into the water of the gill boundary layer (Fig. 1B). Rhcg1 was located only in the interlamellar junction of the gill, where it was apically expressed on mitochondrion-rich cells (MRCs). The substitution of NH_4^+ for K^+ as the substrate of basolaterally expressed NKA in the MRCs may be utilized in unison with Rhcg1 (Fig. 1C) to transport ammonia out of the filament epithelium (Nakada et al., 2007a).

Subsequent studies have identified Rh glycoprotein orthologs in multiple saltwater (Braun and Perry, 2010; Nakada et al., 2010; Rodela et al., 2012) and freshwater (Hung et al., 2007; Nakada et al., 2007b; Nawata et al., 2007; Tsui et al., 2008) species of fish, although the localization of branchial expression varies somewhat between species. Based on such investigations, separate models of branchial ammonia excretion have been developed for fish in saltwater and freshwater environments.

Saltwater models of branchial ammonia excretion

Based largely on the previously mentioned work of Nakada et al. (2007a), Weihrauch et al. (2009) proposed a model for ammonia excretion across the branchial epithelium of marine fishes (Fig. 2). Really, two models are presented, one for pavement cells and one for MRCs. For pavement cells, the model involves basolaterally located Rhbg and apically located Rhcg2 as proposed by Nakada et al. (2007a), but adds that significant diffusion of NH_3 and NH_4^+ likely occurs across the shallow paracellular junction of gill epithial cells in marine fishes. Weihrauch et al. (2009) suggest that since pavement cells comprise the majority (greater than 90%) of the surface area of the marine fish gill, they likely provide the dominant route of branchial ammonia excretion. The proposed MRC model is primarily focused on the transport of NH_4^+ into the MRCs via basolaterally located NKA and NKCC

and excreted as NH_4^+ substituted for H^+ as the substrate of apically located Na^+/H^+-exchanger (NHE-2) or as NH_3 by Rhcg1 (Weihrauch et al., 2009).

Nawata et al. (2010) examined the regulation of Rh glycoproteins and ammonia excretion in pufferfish exposed to high environmental ammonia (HEA). Following an initial influx, ammonia excretion resumed and was accompanied by downregulation of Rhbg mRNA and upregulation of Rhcg1 mRNA, suggesting a mechanism to prevent the backflow of ammonia across pavement cells, while increasing excretion across MRCs. Nawata et al. (2010) speculated that during HEA, as the NH_3 gradient is unfavorable for passive diffusion, ammonia excretion is likely driven by active mechanisms (basolaterally located NKA and NKCC-1, as well as apical H^+-ATPase and NHE-3) available in the MRCs (Fig. 3). In this model, it is assumed that most NH_4^+ is deprotonated prior to excretion via apical Rhcg1. The backflow of NH_3 is further prevented by combination with H^+ excreted by H^+-ATPase or NHE-3 (Nawata et al., 2010).

Freshwater models of branchial ammonia excretion

In freshwater fish the excretion of ammonia appears to be associated with the uptake of Na^+ and excretion of H^+ and is likely dependent upon multiple Rh glycoproteins (Figs 4, 5; Weihrauch et al., 2009; Wright and Wood, 2009). Weihrauch et al. (2009) propose a model utilizing an unspecified combination of Rh glycoproteins in the transcellular movement of ammonia across the gill epithelium and requires acidification of the gill boundary layer by H^+ excretion and CO_2 hydration to maintain a favorable ammonia diffusion gradient (Fig. 4). While diffusion of NH_3 may occur across the paracellular junction in freshwater fish, NH_4^+ is not likely to cross due to the depth and tightness of the junction (Weihrauch et al., 2009).

A similar model proposed by Wright and Wood (2009) provides predictions regarding the Rh glycoproteins likely involved in freshwater branchial ammonia transport that are marginally more specific (Fig. 5). In this model Rhag located on the red blood cells (RBC) are utilized to transport ammonia across the RBC membrane into the plasma and basolaterally located Rhbg facilitates transport into the gill epithelium. Apical Rhcg (variant not specified) facilitates the outward diffusion of NH_3 into the gill boundary layer water, where acid trapping (combination with H^+ to form NH_4^+) is utilized to maintain a favorable diffusion gradient, as described above.

Hagfishes

Among the extant craniates, the hagfishes (Class Myxini) are the most ancient representatives, with fossil evidence supporting a highly conservative evolutionary lineage beginning almost 500 million years ago (Forey and Janvier, 1993). Hagfishes, along with lampreys, are jawless fishes. The phylogenetic position of hagfishes and lampreys relative to the jawed vertebrates (Gnathostoma) is the subject of much controversy, as there is disagreement between predictions drawn from molecular and phenotypic evidence (Near, 2009). Recent microRNA (miRNA) analysis supports a monophyletic grouping of hagfishes and lampreys into Cyclostomata (Heimberg et al., 2010), while phenotypic data suggest a paraphyletic grouping as agnathans (Janvier, 1981). While perhaps adding to confusion surrounding the evolution of hagfishes, the controversy further illuminates their value as essential model organisms for studies of early vertebrate evolution (Escriva et al., 2002; Near, 2009; Takechi et al., 2011).

Despite a developing interest in hagfishes as model organisms and an economically important international fishery (Martini et al., 1997; Powell et al., 2005), little is known

regarding the ecology, life history, and physiology of the Atlantic hagfish (Currie and Edwards, 2010; Weihrauch et al., 2009). Only recently have observations of embryos of the inshore hagfish, *Eptatretus burgeri* (Ota et al., 2007), and examination of the seasonal variations in reproductive steroids of the Atlantic hagfish, allowed a rudimentary understanding of hagfish developmental and reproductive biology (Powell et al., 2004; Powell et al., 2005). In contrast to most vertebrates, hagfishes are marine osmoconformers and were assumed to be ionoconformers (Evans, 1984). However, recent investigation of ionoregulation in Pacific hagfish suggests that while hagfish do ionoconform in regard to Na^+ and Cl^-, they appear to regulate plasma [Ca^{2+}] and [Mg^{2+}] to about half that of seawater (Sardella et al., 2009). Atlantic hagfish are important players in the substrate turnover and nutrient recycling of ocean-floor ecosystems (Lesser et al., 1996) as they spend significant amounts of time burrowed in the anoxic substrate and feed opportunistically on marine carrion (Martini et al., 1997; Strahan, 1963). These behaviors create the potential for exposure to conditions of hypoxia or even anoxia, low pH, elevated CO_2 and most relevantly, HEA (Braun and Perry, 2010; Weihrauch et al., 2009). Understanding the physiological adaptations that enable Atlantic hagfish to tolerate such extreme conditions may provide insight to the evolutionary history of ammonia excretion mechanisms in the invertebrate to vertebrate transition. In the context of comparative physiology, such knowledge could be applied to develop solutions to the problems suffered by less tolerant organisms exposed to similar extreme conditions (Wright and Wood, 2012).

Ammonia excretion in hagfishes

Evans (1984) evaluated Na^+/H^+ exchange as a mechanism of acid-base regulation in Atlantic hagfish and discovered that hagfish exposed to Na^+-free seawater underwent no

change in ammonia excretion rates, suggesting that the $Na^+/NH4^+$ exchange demonstrated in various freshwater and saltwater teleosts may not be utilized in Atlantic hagfish. However, the apparent presence of Na^+/H^+ exchange in the osmoconforming and ionoconforming Atlantic hagfish led Evans (1984) to suggest that acid-base regulation evolved before osmoregulation and the invasion of freshwater by vertebrates. Subsequent work confirmed Na^+/NH_4^+ exchange as a mechanism of acid-base regulation in the Atlantic hagfish and suggested the involvement of an apical NHE in the gill (McDonald et al., 1991), which was supported at the molecular level by Edwards et al. (2001). Multiple studies have identified and localized NKA in hagfish MRCs (Bartels, 1988; Choe et al., 1999; Mallat et al., 1987). Further investigation of ion-transport in Pacific hagfish resulted in the identification and localization of H^+-ATPase, NKA, and NHE2 all in single MRCs (Tresguerres et al., 2006) and suggested involvement in acid-base regulation (Tresguerres et al., 2007). Thus, it is thought that the hagfishes are unique among aquatic craniates and all studied vertebrates in possessing a single MRC utilized for both acid and base excretion and that MRCs are devoted to acid-base regulation and not ionoregulation (Brauner and Baker, 2009; Wright, 2007). Hagfish MRCs are distributed singularly, sandwiched between pavement cells and apparently have a tight paracellular junction (Mallat et al., 1987) that would not likely allow the leaking of NH_4^+ described in other marine fishes (Wright, 2007). Although the ion-transporters (NKA, NKCC, NHE) implicated in ammonia excretion by teleost fishes are also present in hagfishes, the evidence against Na^+/NH_4^+ exchange (Evans, 1984) in the Atlantic hagfish only emphasizes the need to investigate Rh glycoproteins in hagfishes.

Experiments conducted by Braun and Perry (2010) examined the potential role of Rh glycoproteins and urea transporter (UT) proteins in nitrogen excretion in the Pacific hagfish

(*Eptatretus stoutii*). Ammonia excretion rates and plasma ammonia levels were analyzed following either exposure to HEA or injection with NH_4Cl (excluding treatments related to urea). Exposure to 20 mM HEA initiated an influx of ammonia (almost 50 μmol N g^{-1} h^{-1}), which diminished gradually over 9 hours and was reversed to an efflux (over 10 μmol N g^{-1} h^{-1}) when fish were returned to normal seawater. Despite ammonia uptake, the consequent increases in plasma ammonia levels were limited, suggesting either ammonia metabolism to urea or the excretion of ammonia against a gradient created by HEA, each of which is supported by increased plasma urea and decreased ammonia uptake rates over time during HEA. Injections of NH_4Cl resulted in impressive ammonia excretion rates (over 40 μmol N g^{-1} h^{-1} during the three hours following injection). However, ammonia excretion rates were calculated for a relatively coarse spatial resolution (3 hour intervals) and observations were terminated too early to determine if excretion rates in ammonia loaded hagfish would equilibrate or return to basal levels. The study did not investigate Rh glycoproteins at the molecular level, but instead utilized heterologous antibodies raised against teleosts to detect Rhag, Rhbg and Rhcg1 protein expression, while only Rhbg and Rhcg1 were immunolocalized due to non-specific immunoreactity in Rhag immunoblots. Still, protein expression was not quantified for any Rh glycoproteins and the use of heterologous antibodies may have yielded inconclusive immunolocalization (Braun and Perry, 2010).

To further develop the evolutionary context of Rh glycoproteins and their role in the regulation of ammonia excretion in craniates, the investigation of Rh isoforms in the Atlantic hagfish would provide valuable information regarding the evolutionary history of ammonia excretion across the invertebrate to vertebrate transition. Recent molecular cloning of the Rhcg and Rhbg isoforms in the Atlantic hagfish (Edwards and Walsh, unpublished) has

allowed the development of hagfish specific probes for *in situ* hybridization (Edwards, unpublished) and enables the subsequent development of hagfish specific primers and homologous antibodies for the quantification and localization of both molecular and protein expression.

Purpose of study

We hypothesize that the expression of Rh glycoprotein mRNA and protein are upregulated in response to experimentally elevated plasma ammonia concentrations in the Atlantic hagfish. We propose three objectives to evaluate this hypothesis:

1. Determine the effect of experimentally elevated plasma ammonia concentration on ammonia excretion in Atlantic hagfish
2. Quantify Rh glycoprotein mRNA expression in gill and skin tissues in response to experimentally elevated plasma ammonia concentrations
3. Develop a homologous Rhcg antibody for the quantification and localization of protein expression

We predict that experimentally elevated plasma ammonia levels will cause increased ammonia excretion correlating with increased expression of Rh glycoprotein mRNA transcripts.

Methods

RNA isolation and cDNA synthesis

Total RNA was isolated from gill and skin tissues of *M. glutinosa* by homogenization in 1 ml of TRI-reagent (Molecular Research Center, Inc. Cincinnati, Ohio) for every 50-100 mg of tissue, followed by separation and extraction using bromochloropropane, then precipitation in isopropanol. The resulting RNA precipitate was pelleted by centrifugation at 12,000 *g* for 8 minutes at room temperature and washed using 75% ethanol. The washed RNA pellet was solubilized in HyClone molecular grade water (Thermo Fisher Scientific, Rockford, IL). The purity and concentration of solubilized total RNA were assessed by measuring sample absorbance at 260/280 nm by spectroscopy (Nanodrop 2000, Thermo Fish Scientific, Rockford, IL). First strand complementary DNA (cDNA) was synthesized from 5 ug total RNA using Superscript III reverse transcriptase enzyme primed with an Oligo-dT primer (Invitrogen, Carlsbad, CA).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Previous work by Edwards and Walsh (unpublished) utilized a combination of RT-PCR and RACE (rapid amplification of cDNA ends) PCR to clone the hagfish Rhcg (Fig. 6; GenBank accession number GU73440) and Rhbg (Fig. 7; GenBank accession number GU73441). Atlantic hagfish specific primers (Table 1) were designed based on these sequences to allow amplification of Rhbg and Rhcg cDNAs across a predicted, non-

conserved intron–exon boundary. Primers were also designed to target ribosomal protein 18S (Table 1) for use as an endogenous control in the relative quantitation of Rhcg and Rhbg mRNA by subsequent quantitiative RT-PCR (qRT-PCR). A hot start PCR (Platinum *Taq*, Invitrogen Carlsbad, CA) of 25 ul volume was incubated using a MJ Mini personal thermal cycler (Bio-Rad, Hercules, CA). PCR reagent concentration for all reactions was 3 mmol l^{-1} MgCl, 200 umol l^{-1} dNTP mix, 10mmol l^{-1} for each primer and 1.25 units Platinum *Taq*. An initial incubation at 94°C for 2 minutes allowed activation of the Platinum *Taq* polymerase. This was followed by 36 cycles of melting at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 60 seconds, and a final extension time of 72°C for 10 min. The PCR products were analyzed by gel electrophoresis (1% agarose in a 1X TBE buffer, 45mM Tris borate, 1 mM EDTA) stained with ethidium bromide and visualized by trans-illumination with UV light.

Molecular cloning and sequencing

Amplicons of appropriate sizes (Table 1) were ligated into the pCR2.1-TOPO plasmid vector (Invitrogen, Grand Island, NY) and transformed into competent One Shot® TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA). Cells were plated onto LB agar plates containing 50 μg ml^{-1} ampicillin (Thermo Fisher Scientific, Rockford, IL) and 40 μg ml^{-1} X-gal (Promega, Madison, WI) and incubated at 37°C overnight. Positively transfected clones were selected by blue/white colony screening and cultured in LB broth (5 μg ml^{-1} ampicillin). Plasmid DNA was isolated using a FastPlasmid miniprep kit (5 Prime Inc., Gaithersburg, MD) and analyzed by restriction enzyme digest using EcoR1 and agarose gel electrophoresis. Clones containing an insert were combined with forward or reverse sequencing primers and sequenced on an ABI PRISM 3100 genetic

analyzer (Applied Biosystems, Foster City, CA) at Mount Desert Island Biological Laboratory, Salisbury Cove, ME to confirm that primers were specific to the appropriate Rh glycoprotein cDNAs.

Phylogenetic analysis

Relevant Rh glycoprotein sequences were retrieved from the GenBank database using the standard BLAST algorithms available from the National Center for Biotechnology (NCBI). An alignment of translated amino acid sequences was carried out using the ClustalW algorithm (Thompson et al., 1994) in MacVector 12.6 (MacVector, Cary, NC). Phylogenetic reconstruction of the multiple sequence alignment used a neighbor joining algorithm and bootstrap calculation (1000 replications) with Poisson-corrected distances and proportionally distributed gaps. The resulting tree was rooted to the *Chlamydomonas reinhardtii* Rhp1.

Experimental design and animals

A sample of 79 Atlantic hagfish, ranging from 40-115 g wet mass (see Table 2), were obtained from the Bay of Fundy by commercial fisherman and maintained in a covered flow-through aquarium with 7 – 12˚C running seawater at Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, Maine. All hagfish were held without feeding for at least 2 weeks prior to experimentation. Individuals were randomly assigned to control (N=38, sham-injected) or experimental (N=38, ammonia-injected) groups. Samples were also collected from individuals that were subjected to identical conditions, but were not injected (N=3) to provide basal physiological data. Experiments were conducted to allow repeated collection of water samples following injections and terminal tissue sampling from three to five individuals of each group over 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hour durations.

All animal research activities were approved by institutional animal care and use committees (IACUC) at both MDIBL and Appalachian State University.

Injections

Individuals were placed into continuously circulated, aerated seawater in 1 L darkened containers for an acclimation period of 12 hours. Containers were housed in a continuously circulating seawater bath to maintain consistent water temperature (7 - 12˚C). Prior to injections, individuals were lightly anesthetized using 40 mg ml^{-1} MS-222 (Finquel, Argent Chemical Laboratories, Redmond, Washington) to reduce slime production during handling. The protocol of Braun and Perry (2010) was followed to experimentally elevate plasma ammonia concentrations by injection of 100 mM NH_4Cl dissolved in seawater at a pH 7.8 into the caudal subcutaneous sinus. Injection volumes were calculated to elevate plasma ammonia concentration to an estimated 10 mM NH_4Cl, by assuming 30% of whole organism mass to be extracellular fluid (Braun and Perry, 2010). Control individuals were injected with a seawater sham (~34 ppt) at a pH 7.8 into the caudal subcutaneous sinus, with sham volumes calculated identically to NH_4Cl injections. Following injection, individuals were inverted several times to insure mixing of the injected bolus into circulating plasma prior to returning to their respective containers.

Water sampling

Seawater within containers remained aerated throughout the duration of each experiment, but circulation was stopped prior to returning injected individuals to containers to allow static water sampling. Water samples (2 ml) were obtained from each container just prior to and during hagfish occupancy at all time-points occurring within the respective experimental duration (0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours following injection). Water

samples were frozen and stored at -20˚C for the subsequent determination of total ammonia concentrations.

Sacrifice and tissue sampling

Experiments were terminated after the appropriate duration by a lethal dose (80 mg ml^{-1}) of MS-222 (Finquel) administered to each container. Following the termination of life, 1-2 ml of blood was drawn from the caudal sinus of each hagfish using a heparinized needle and syringe. Blood samples were then centrifuged at 12,000 *g* for 1 minute to separate plasma. The plasma layer was removed, snap-frozen in liquid nitrogen and stored at -80˚C prior to ammonia determination. Gill, skin, intestine (upper and lower), and kidney tissues were collected and either snap-frozen in liquid nitrogen and stored at -80˚C for subsequent RNA and protein isolation or fixed in 4% paraformaldehyde in phosphate buffer for immunohistochemical analysis.

Water ammonia profile

Water samples were analyzed, in triplicate, to determine the total ammonia concentration (T_{Amm}) by a micro-plate modification of the phenol-hypochlorite method (Weatherburn, 1967). It should be noted that the determination of total ammonia includes both NH_4^+ and NH_3, thus no quantitative distinction will be made. Standard solutions ranging from 5 to 200 mM NH_4Cl were prepared in seawater. Sample absorbance was measured at 640 nm with a Bio-Tek Powerwave plate reader (Thermo Fisher Scientific, Rockford, IL) and VersaMAX plate reader (Molecular Devices, Sunnyvale, CA). Net flux rates (μmol kg^{-1} h^{-1}) of T_{Amm} were calculated as:

$$J_{Amm} = ([T_{Amm}]_i - [T_{Amm}]_f) \times V / (\Delta t \times M) \quad (1)$$

where $[T_{Amm}]_i$ and $[T_{Amm}]_f$ are initial and final T_{Amm} concentrations (μmol l^{-1}) in the water, V is the volume of water (l) in the container during the flux period, Δt is the time elapsed (h) during the flux period and M is the mass of the fish (kg). A positive J_{Amm} indicates net excretion and a negative value indicates net uptake of ammonia. Data analysis was performed with Microsoft Excel 2010.

Plasma ammonia profile

A 200 μl aliquot of each plasma sample was deproteinized in two volumes of 8% perchloric acid, vortexed and centrifuged at 16,000 *g* for 10 minutes (4°C). The supernatant was neutralized with saturated $KHCO_3$ and centrifuged at 16,000 *g* for 10 minutes (4°C). Using the final deproteinized supernatant, ammonia concentrations (μg ml^{-1}) were established in triplicate using a micro-plate modification of the L-glutamate dehydrogenase assay (Sigma AA0100, St. Louis, MO). The absorbance of each sample was measured at 340 nm following incubation (~ 5 minutes) with the ammonia assay reagent and again approximately 5 minutes after the addition of L-glutamate dehydrogenase enzyme. Sample absorbance was measured with a VersaMAX plate reader (Molecular Devices, Sunnyvale, CA). The mean total ammonia concentration for each sample was calculated using Microsoft Excel 2010.

Quantitative RT-PCR (qRT-PCR)

The relative expression of Rhcg and Rhbg mRNA was quantified in gill and skin tissues of sham-injected and ammonia-injected individuals using the previously mentioned Atlantic hagfish specific primers (Table 1). The quantification of Rhcg and Rhbg mRNA expression for individuals in experiments of durations longer than 8 hours following injection were deemed unnecessary, as plasma ammonia concentrations and the net flux of ammonia

were not significantly different between sham-injected and ammonia-injected individuals in subsequent time-points. The ribosomal protein 18S was used as an endogenous control for the $2^{(-\Delta\Delta Ct)}$ method (Pfaffl, 2001). Each sample was analyzed in triplicate using 25 ng of template cDNA, 70 nmol of each forward and each reverse primer, 0.5 µl of ROX reference dye diluted 1:10 and SYBR® GreenER SuperMix-UDG (Invitrogen, Carlsbad, CA) in a total volume of 25 µl. Amplification of the desired product was confirmed using melt curve analysis. Relative mRNA expression in ammonia-injected individuals was normalized to that of the sham-injected individuals from the same experiments (time durations). Threshold cycle (C_t) was determined for each sample using ABI Systems 7500 software. Analysis of relative mRNA expression using the $2^{(-\Delta\Delta Ct)}$ method (Pfaffl, 2001) was conducted using Microsoft Excel 2010.

Antibody production

Commercially synthesized *Myxine* specific Rhcg peptide was developed from Rhcg amino acid sequence 419-434 (CYEDRAYWEVPEEEVTY) (Bio-Synthesis, TX). The polyclonal Rhcg antibody was prepared in-house at Appalachian State University. A single New Zealand white rabbit (*Oryctolagus cuniculus*) was inoculated with a total of 5 injections of 1-1.5 mg (0.5 ml, 2-3 mg/ml) of purified peptide over a 15-week period. Following the initial subcutaneous injection, three subsequent "booster" injections were intramuscularly administered at a single site. Blood was collected by 4-5 ml blood draws 7 days after each immunization to determine antibody titer and a terminal bleeding at the end of the 15-week immunization period to harvest immune serum. All blood was clotted overnight in sterile glass tubes at 4°C, followed by two centrifugations (10 min., 10,000 *g*) to separate immune serum. Antibody titer was assessed by enzyme-linked immunosorbent assay (ELISA).

Western blotting

Protein was isolated from frozen hagfish gill tissues. Tissues were placed in buffer (0.18g Tris-base, 4.28g sucrose, 0.5ml 100mM EDTA, pH 7.8) and homogenized in polypropylene tubes on ice. The homogenate was centrifuged at 4˚C to separate a protein fraction. The protein fraction was removed and total protein concentration was quantified using a BCA (bicinchoninic acid) protein assay (Thermo Scientific, Rockford, IL). Protein samples (50µg) were loaded in Tris-HEPES polyacrylamide gels (NuSep, Homebush, Australia) and separated by SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis). Separated proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked overnight at 4°C in 5% blotto (5% nonfat dry milk powder in 0.1M Tris-buffered saline with 0.2% Tween-20). Membranes were then incubated in 5% blotto (negative control), homologous Rhcg antibody (1:5000) in 5% blotto (positive control), or homologous Rhcg antibody preabsorbed 1:10 and 1:20 with purified Rhcg peptide (1:5000) in 5% blotto overnight at room temperature. Following three washes (0.1M Tris-buffered saline with 0.2% Tween-20 (TBST)), membranes were incubated with HRP (horseradish peroxidase)-conjugated goat anti-rabbit antibody (1:10,000) and Precision Protein StrepTactin-HRP conjugate (Bio-Rad, Hercules, California, USA) in TBST at room temperature for one hour. Unbound secondary antibodies were removed with three additional washes in TBST and a final wash in TBS (no Tween-20). Immunoblotted membranes were then prepared using an enhanced chemiluminescence system (Bio-Rad) and developed on Amersam Hyperfilm ECL X-ray film (GE Healthcare, Little Chalfont, United Kingdom).

Statistical analysis

Data are presented as the mean ±1SEM (one standard error of the mean). Hypotheses were tested using an appropriate two-sample *t*-test (α=0.05; Student's *t*-test if variances were homoscedastic; Welch's *t*-test if variances were heteroscedastic) for the comparison of two means within each time-point.

Results

RT-PCR and phylogenetic analysis

RT-PCR using Atlantic hagfish specific primers yielded a single product of the expected size (Table 1) for each target primer set (ribosomal protein 18S, Rhcg, and Rhbg) in gill and skin tissues (Fig. 8). No detectable product was produced in the RT-PCR reactions containing no cDNA template (no-template control, NTC, Fig. 8).

Phylogenetic reconstruction (Fig. 9) produced separate clades for Rhcg and Rhbg lineages and, with the exception of the stickleback (*Gasterosteus aculeatus*) Rhp2 grouping with Rhbg, the Rhp isoforms group ancestrally. Rhp1 from the crab, *Carcinus maenas*, was grouped ancestrally to Rhcg and Rhbg clades, as well as the zebrafish (*Danio rerio*) Rhp2.

Water ammonia profile

Cumulative total ammonia was significantly elevated ($P<0.05$) in the environmental water of individuals injected with 100 mM NH_4Cl at all observed time points following injection, reaching 2,212.79±11.00 μmol kg^{-1} by 24 hours post-injection (Fig. 10). Cumulative ammonia concentrations in the environmental water of control individuals increased over time to 637.78±15.85 μmol kg^{-1} by 24 hours following sham injection.

Efflux of ammonia was observed across all time-points for both sham and ammonia-injected hagfish. Individuals injected with ammonia exhibited a significantly increased ($P<0.05$) rate of ammonia efflux (relative to sham-injected individuals) up to 4 hours post-

injection. The largest efflux (2895.06±496.29 μmol kg^{-1} h^{-1}) occurred within the 0.25 hours following injection (Fig. 11). There were no significant differences in ammonia efflux between sham and ammonia-injected groups from 4-6 hours post-injection and throughout all subsequent time-points.

Plasma ammonia profile

The concentration of total ammonia detected in the plasma of ammonia-injected hagfish was significantly higher than in sham-injected individuals at all times points, except 8 hours post-injection (Fig. 12). Plasma ammonia concentrations were most elevated at 0.25 hours following injection (11.89±1.56 μg ml^{-1}), but generally decreased over time relative to concentrations observed in sham-injected individuals. Plasma ammonia levels were not significantly different between groups at 8 hours post-injection. However, plasma ammonia did significantly increase (P=0.008) again in ammonia-injected hagfish to 2.29±0.41 μg ml^{-1} at 12 hours post-injection (Fig. 12).

qRT-PCR

The relative expression of Rhcg mRNA was significantly altered (P<0.05) in the gill tissues of ammonia-injected individuals at all time points (Fig. 13). Rhcg mRNA expression was increased 5.2±-fold and 7.9±-fold above the sham-injected group expression at 0.25 hours and 0.5 hours after injection, respectively. From 1 hour post-injection onward, Rhcg mRNA expression in ammonia-injected individuals was less than half that of sham-injected individuals.

Expression of Rhcg mRNA in skin tissue was significantly altered in ammonia-injected individuals, with a 6.17±0.0-fold elevation above the sham injected group at 0.5 hours (P=0.002) and less than one fifth of sham-injected expression at 4 hours

(P=0.0000165) post-injection (Fig. 14). The differences in Rhcg mRNA expression in skin tissue at the other time-points were not statistically significant.

The expression of Rhbg mRNA in the gill tissue of ammonia-injected hagfish was significantly elevated relative to the sham-injected group at 0.25 (2.16±0.58-fold, P=0.012) and 0.5 hours (2.24±0.40-fold, P=0.015) post-injection (Fig. 15). Rhbg mRNA expression in the ammonia-injected group was less than half that of sham-injected group expression at 1, 4, and 8 hours post-injection (P<0.005). Gill expression of Rhbg mRNA was not significantly different between treatments at 2 hours post-injection. Expression of Rhbg mRNA in skin tissue was not reliably detectable using the methods described above and data are not shown.

Western blotting

Atlantic hagfish gill protein immunoblots treated with no primary antibody (negative control) did not present the immunoreactive protein at 37 kDa (Fig. 16A). The positive control (Fig. 16B) had a prominent band at approximately 37 kDa and this band is diminished in immunoblots treated with Rhcg antibody preabsorbed with peptide at 1:10 (Fig. 16C) and 1:20 (Fig. 16D) ratios.

Discussion

This is the first study to quantify Rhcg and Rhbg mRNA expression in the Atlantic hagfish, *Myxine glutinosa*. Our primary objective was to investigate the potential role of Rh glycoproteins in ammonia excretion by examining differential expression of Rh glycoprotein mRNA between hagfish with experimentally elevated plasma ammonia levels and hagfish with baseline plasma ammonia levels. We hypothesized that increased plasma ammonia concentrations would result in an efflux of ammonia associated with increased expression of Rhcg and Rhbg mRNA transcripts, which would then be translated into proteins involved in facilitating the transport of ammonia. The presented results provide support for our hypothesis. The injection of ammonia elevated plasma ammonia concentrations (Fig. 12) and resulted in a striking and nearly immediate efflux of ammonia (Fig. 11) that correlates with the early upregulation of Rhcg in gill (Fig. 13) and skin (Fig. 14), as well as Rhbg in gill (Fig. 15).

Evolutionary context of hagfish Rh glycoproteins

Phylogenetic reconstruction (Fig. 9) of Atlantic hagfish Rhcg and Rhbg lineages relative to Rhcg, Rhbg, Rhp2 and Rhp1 isoforms in a small selection of other organisms corroborates the phylogenetic position of hagfishes as basal to the vertebrates, suggesting that hagfish Rhcg and Rhbg are ancestral to their respective isoforms in the higher vertebrates. The primitive isoform Rhp2 in the zebrafish (*Danio rerio*), although present in a

teleost fish, appears to be ancestral to the hagfish Rhcg and Rhbg, as would be expected given the results of Huang and Peng (2005). Rhp1 and Rhp2 have not yet been identified in the Atlantic hagfish.

Ammonia excretion in response to elevated plasma ammonia

The cumulative ammonia profile (Fig. 10) of ammonia-injected hagfish illustrates an obvious accumulation of environmental ammonia relative to that of sham-injected individuals, indicating an outward flux of ammonia (Fig. 11). Sham-injected hagfish also demonstrate an outward flux and accumulation of ammonia, although to a much lesser degree than ammonia-injected hagfish (Figs 10, 11).

The highest rates of ammonia efflux occurred within the first hour following injection (Fig. 11), suggesting that Atlantic hagfish are either readily equipped for remarkable ammonia excretion (more the 10X that of sham-injected fish) or that the necessary protein components may be assembled very rapidly to facilitate increased ammonia excretion. Initial concerns were raised that injected NH_4Cl might be leaking out from the site of injection; however, these concerns were alleviated as no leakage was observed following injection as outlined in the methods, but with the addition of colored food dye. McDonald et al. (1991) reported ammonia excretion rates of nearly 1400 μmol N kg^{-1} h^{-1} over the first 0.5 hour following 4 mmol kg^{-1} infusion of ammonia as $(NH_4)_2SO_4$, which was a 7-fold increase over resting ammonia excretion rates. For comparison, ammonia excretion rates in the current study, calculated over the first 0.5 hour following injection, were 1558.766±89.786 μmol N kg^{-1} h^{-1} (nearly a 7.5-fold elevation from control ammonia excretion rates during the same period) following a 3 mmol kg^{-1} infusion of ammonia as NH_4Cl. This suggests a slightly higher rate of excretion in the current study, even with the infusion of a lesser ammonia load.

Ammonia excretion rates in ammonia-injected hagfish gradually diminished to sham-injected levels by 6 hours post-injection, with a parallel reduction in plasma total ammonia concentrations by at least 8 hours post-injection (plasma samples were not obtained at 6 hours post-injection). However, the maximum accumulated total ammonia concentration in ammonia-injected hagfish, occurring at 24 hours post-injection, was 2.2 mmol kg^{-1}, almost 74% of the 3 mmol kg^{-1} load introduced by injection. The net remaining 0.8 mmol kg^{-1} of total ammonia was not excreted into the environmental water, however, was apparently eliminated from the plasma. To account for this part of the ammonia load, we suggest multiple possible fates. Wilkie and Wang (1999) reported significantly elevated total ammonia concentrations in the muscle tissue of another agnathan fish, the sea lamprey (*Peromyzon marinus*), relative to plasma concentrations, suggesting that the muscles may serve as a reservoir to reduce circulating plasma ammonia concentrations. It is possible that a similar mechanism could be utilized in the Atlantic hagfish. Braun and Perry (2010) demonstrated elevated urea excretion in Pacific hagfish injected with ammonia and exposed to HEA. Although it is not known whether Atlantic hagfish possess the enzymes involved in the metabolism of ammonia into urea, it is possible that the remaining ammonia load in the current study could have been converted to urea and excreted. However, urea concentrations were not measured in the current study.

Prior to this study little was known about the Atlantic hagfish's ability to excrete ammonia (Evans, 1984; McDonald et al., 1991); given its known burrowing and feeding behaviors (Martini et al, 1997) the ability to rapidly excrete ammonia in less than favorable conditions would likely be advantageous and thus not surprising. Atlantic hagfish spend the majority of their life burrowed in hypoxic (or even anoxic) ocean-floor substrate and may

remain there for days at a time (Strahan, 1963). Although gut-content analysis has shown that Atlantic hagfish may feed primarily on shrimp and other invertebrates (Shelton, 1978), they are known to opportunistically feed on the carcasses of whales and teleost fishes. Hagfishes are able to tear away and consume exterior portions of a carcass, but are perhaps more likely to enter through an orifice to feed while burrowed in the rotting flesh (Strahan, 1963). Burrowing in substrate and feeding within carcasses probably reduces gas exchange and is likely to upset acid-base balance (Brauner and Baker, 2009). Conditions within a carcass likely include low O_2, high CO_2, low pH, and HEA (Currie and Edwards, 2010; Weihrauch et al., 2009). Extended exposure to hypoxic/anoxic conditions is allowed by multiple physiological adaptations in the hagfishes, including exceptionally low metabolic rates and energetic requirements, as well as cutaneous respiration (Lesser et al., 1996). Due, at least in part, to a common mechanism of neurotoxicity between hypoxia and ammonia (glutamine excitotoxicity), fishes that are tolerant of hypoxia are often ammonia tolerant, as well (Walsh et al., 2007). Pacific hagfish are apparently tolerant to both internal ammonia loads and HEA exposure, surviving more than 72 hours of exposure to 100 mM NH_4Cl (Braun and Perry, 2010). Atlantic hagfish in the current study were tolerant of a 3mmol kg^{-1} infusion of ammonia as NH_4Cl, apparently eliminating the internal ammonia load and surviving for 24 hours post-infusion.

Rh glycoprotein mRNA expression in gill tissue

We hypothesized an increase in gill Rhcg and Rhbg mRNA expression in response to elevated plasma ammonia, if these isoforms indeed do have a role in ammonia excretion. Our results show increased Rhcg and Rhbg mRNA expression in ammonia-loaded fish prior to 1 hour post-injection. This is not necessarily surprising, as elevated plasma ammonia may

serve as a stimulus for the increased production of Rh glycoproteins and may suggest that the expression of Rh glycoprotein mRNA is tightly regulated in response to ammonia infusion. Evidence presented by Nawata and Wood (2008) supports the upregulation of branchial Rhcg1, Rhcg2, and Rhbg mRNA expression in response to ammonia infusion in freshwater rainbow trout. However, they suggest that a combination of elevated plasma ammonia and a temporary pulse of cortisol may be key to Rh mRNA regulation. Atlantic hagfish plasma cortisol levels were not assessed in the current study due to a long-standing controversy regarding the active corticosteroid in hagfish (Weisbart and Idler, 1970). Atlantic hagfish Rhcg mRNAs were downregulated at 1 hour and subsequent time-points in ammonia-injected hagfish (Fig. 13), despite elevated plasma ammonia concentrations and ammonia excretion rates up to 4 hours following ammonia-injection. Regulation of branchial Rhbg mRNA expression (Fig. 14) followed a similar pattern, with significant upregulation in ammonia-injected hagfish at 0.5 hours post-injection, followed by either downregulation or no significant difference in expression relative to sham-injected hagfish at all subsequent time-points. This pattern of regulation suggests an immediate response to elevated plasma ammonia that is subsequently altered in response to additional factors. Among the possible stimuli inducing the downregulation of Rh mRNA expression is HEA (Nawata et al., 2010). Rh glycoproteins may transport ammonia bi-directionally (Marini et al., 2000) and thus could potentially allow back (inward) flux of ammonia during a reversed diffusion gradient caused by HEA. As mentioned above, this hypothesis was evaluated by Nawata et. al. (2010) in marine pufferfish (*Takifugu rubripes*) exposed to HEA. Pufferfish exposed to 1 mmol l^{-1} HEA downregulated Rhag mRNA expression after 6 hours and downregulated Rhbg mRNAs after 24 hours of exposure. However, the authors did not perform quantification of Rh

protein expression, due to the presence of multiple non-specific bands in western blot analysis. Interestingly, Rhcg1 mRNA expression, as well as that of NKCC1, NKA, NHE3, and H^+-ATPase, was upregulated in response to HEA. This led Nawata et al. (2010) to propose that, after prolonged HEA exposure, ammonia transport may shift from a dependence on passive transport via Rh glycoproteins to active transport by the recruitment of ion-transporters in MRCs (see description of MRC model above and Fig. 3). It is conceivable that accumulated HEA could stimulate downregulation of branchial Rhcg and Rhbg mRNA expression in ammonia-injected hagfish, even while plasma ammonia concentrations and ammonia excretion rates both remain elevated above sham-injected levels as a protective mechanism to reduce the backflow of ammonia as in Nawata et al. (2010).

Quantification of protein expression from individuals in this study was conducted by Edwards (unpublished) using the previously described homologous Rhcg antibody and suggests that upregulated Rhcg mRNA in ammonia-injected hagfish was translated to protein. Rhcg protein expression was significantly elevated ($P<0.05$) by 2 hours post-injection and remained significantly elevated through 8 hours post-injection in ammonia-injected hagfish (Fig. 17). In ammonia-injected hagfish, the highest protein expression (relative to sham-injected individuals) of Rhcg occurred at 8 hours post-injection, after plasma ammonia and ammonia excretion rates were reduced to control levels at (Fig. 17). This suggests that branchial Rhcg mRNA that was upregulated at 0.25 and 0.5 hours post-injection was still being translated into protein at 8 hours post-injection. The mRNA and protein turnover rates of hagfish Rhcg are unknown and thus it is difficult determine the specific temporal relationships between the transcriptional regulation of Rhcg, protein expression and ammonia excretion rates over the time course of this study (Sashaw et al.,

2010). However, the current study and the protein expression analysis discussed above provide the first insights into the regulation of potential Rhcg mediated ammonia excretion from the transcript to protein levels. A possible scenario is that the Atlantic hagfish may be equipped for an immediate response to elevated plasma ammonia by utilizing existing Rhcg glycoproteins, while immediately increasing expression of Rhcg mRNA transcripts to replenish Rh glycoprotein stores prior to actual protein turnover.

Localization of Rhcg in the gill tissue using the same homologous Rhcg antibody (Figs 18A, B) demonstrates a basolateral staining pattern associated with the branchial epithelium closest to the blood margin (Pray, 2013, unpublished thesis). The structure of hagfish gills is rather different than that of teleost gills, but Rhcg expression occurred in the multilayered epithelium of the primary fold, the structural equivalent of the gill filament in teleosts (Elger, 1987). Like the gill filament of marine teleosts, the 'filament' of the Atlantic hagfish has a higher density of MRCs than the lamellae (Choe et al., 1999; Evans et al., 2005).

Rh glycoprotein mRNA expression in skin tissue

Rhcg mRNA expression in the skin (Fig. 14) followed a similar pattern to that seen in the gill tissue (Fig. 12), but with upregulation in ammonia-injected hagfish occurring only at 0.5 hours following injection. Immunolocalization (Figs 18C, D; Pray, 2013, unpublished thesis) and western blotting (Edwards, unpublished) suggest that Rhcg protein is expressed in the skin; however, protein expression was not quantified in the skin due to poor immunoreactivity. However, immunolocalization of Rhcg in the skin (Figs 18C, D) demonstrated a basolateral staining pattern associated with the cutaneous epithelium (Pray, 2013, unpublished thesis).

Among teleost fishes, the gill serves as the primary site of ammonia excretion (Evans et al., 2005). Rhcg mRNA expression data from this study (Fig. 14) suggest that cutaneous routes involving Rh glycoproteins may play a role in ammonia excretion in the Atlantic hagfish. Rh glycoproteins have been identified in the skin of rainbow trout (Nawata et al., 2007), mangrove rivulus (Hung et al., 2007), zebrafish (Shih et al., 2008), and pufferfish (Nawata et al., 2010); all experiments involving exposure to HEA (see Glover et al., 2013, for a recent review of fish skin as a transport epithelium). Hung et al. (2007) suggested that elevated environmental ammonia induces extra-branchial expression of Rh glycoproteins to provide additional pathways for ammonia excretion. Shih et al. (2008) provide evidence for Rhcg1-mediated ammonia excretion by ionocytes in the skin of larval zebrafish, relying on acid trapping H^+-ATPase driven H^+ excretion. Subsequent research (Shih et al., 2013) also implicates Rhbg in ammonia excretion via skin ionocytes.

It was not within the scope of this study to determine the specific contributions of branchial and extra-branchial routes towards overall ammonia excretion, however, we present evidence that Rhcg and Rhbg are expressed in the skin of the Atlantic hagfish and that cutaneous Rhcg mRNA expression may respond to elevated plasma ammonia. In contrast to Rhcg mRNA expression in the gill, significant upregulation of Rhcg mRNA in the skin of ammonia-injected hagfish did not occur until 0.5 hour following injection. Although Rhbg mRNA and protein expression were not quantified, Rhbg mRNA was detected in skin using RT-PCR (Fig. 8). Expression of Rhcg and Rhbg mRNAs were not compared between gill and skin tissues, however, gel analysis of RT-PCR products and comparison of qRT-PCR threshold cycles suggest that mRNA expression may be lower in the skin. This would likely contribute to the difficulty in reliably quantifying Rhbg mRNA in the skin.

Regulation of ammonia excretion in the Atlantic hagfish

Based on the evidence in the current study, branchial ammonia excretion in the Atlantic hagfish likely involves the Rh glycoproteins Rhcg and Rhbg. Immunolocalization evidence suggests that ammonia may enter the near the blood margin of gill epithelium via Rhcg. The localization of hagfish Rhbg is uncertain as a homologous antibody has not been developed and immunolocalization using a heterologous teleost antibody proved inconclusive in Pacific hagfish (Braun and Perry, 2010). Based on the literature reviewed above and the proposed models of both freshwater and saltwater ammonia excretion (Figs 1-5), it is likely that ammonia excretion across the branchial epithelium by an apically located Rh glycoprotein would rely on H^+ excreted by apical H^+-ATPase or NHE to maintain a favorable gradient for passive NH_3 excretion. Evans (1984) presented evidence that implies Na^+/NH_4^+ exchange (especially across the apical membrane) is not significant in the Atlantic hagfish. However, there may be a possibility that, in hagfish, ammonia could cross the basolateral membrane of the branchial epithelium as NH_4^+ displacing K^+ as the substrate of basolaterally located NKCC or NKA (not necessarily relying on the uptake of external Na^+). This hypothesis is speculative and awaits experimental evaluation.

Limitations

The current study provides valuable quantitative evidence of transcriptional regulation of Rh glycoproteins in response to elevated plasma ammonia in Atlantic hagfish. Loss of function experiments with Atlantic hagfish would be useful in confirming the role of Rh glycoproteins in ammonia excretion. However, there are no known chemical inhibitors of Rh glycoproteins and thus loss of function studies require the genetic manipulations (Hwang et al., 2011) available in zebrafish (Shih et al., 2008) and medaka (Liu et al., 2013). The

current study presents the only known homologous antibody for an Rh glycoprotein (Rhcg) in hagfish. Still, the lack of any other homologous antibodies for hagfish Rh glycoproteins remains as a limitation to reliable quantification and localization of Rh protein expression.

Future directions

Molecular cloning should proceed to identify any other Rh isoforms present in the Atlantic hagfish. Identification of Rhp1 or Rhp2 would be of particular value in establishing the role of these primitive Rh isoforms in an organism close to the invertebrate/vertebrate transition (Weihrauch et al., 2009).

Rhcg protein expression (Fig. 17) determined by Edwards (unpublished) in ammonia-injected hagfish remained elevated after plasma ammonia and ammonia excretion rates had returned to control levels. This finding may warrant sub-cellular localization of Rhcg using transmission electron microscopy (TEM) and immunogold labeling, as well as characterization of Rh glycoprotein turnover rates.

Further investigations are required to elucidate the role of hagfish skin in ammonia excretion and to assess what role Rh glycoproteins may have in the regulation of extra-branchial routes of ammonia excretion. Divided chamber experiments allowing the separate evaluation of branchial and extra-branchial ammonia excretion would likely present logistical challenges, but may allow the determination of the specific contribution of the skin in proportion to branchial ammonia excretion.

Extended work involving tissues from the current study should include the quantification of ion-transporter (NHE, NKA, NKCC, and H^+-ATPase) mRNA and protein expression in relation to plasma ammonia concentrations, ammonia excretion rates, and expression of Rhcg and Rhbg mRNA and protein. The development a homologous Rhbg

antibody to be utilized in combination with existing Rhcg, NKA, NHE, and H^+-ATPase antibodies could enable the immunolocalization necessary to develop a reliable model of ammonia transport and excretion in the Atlantic hagfish.

Conclusions

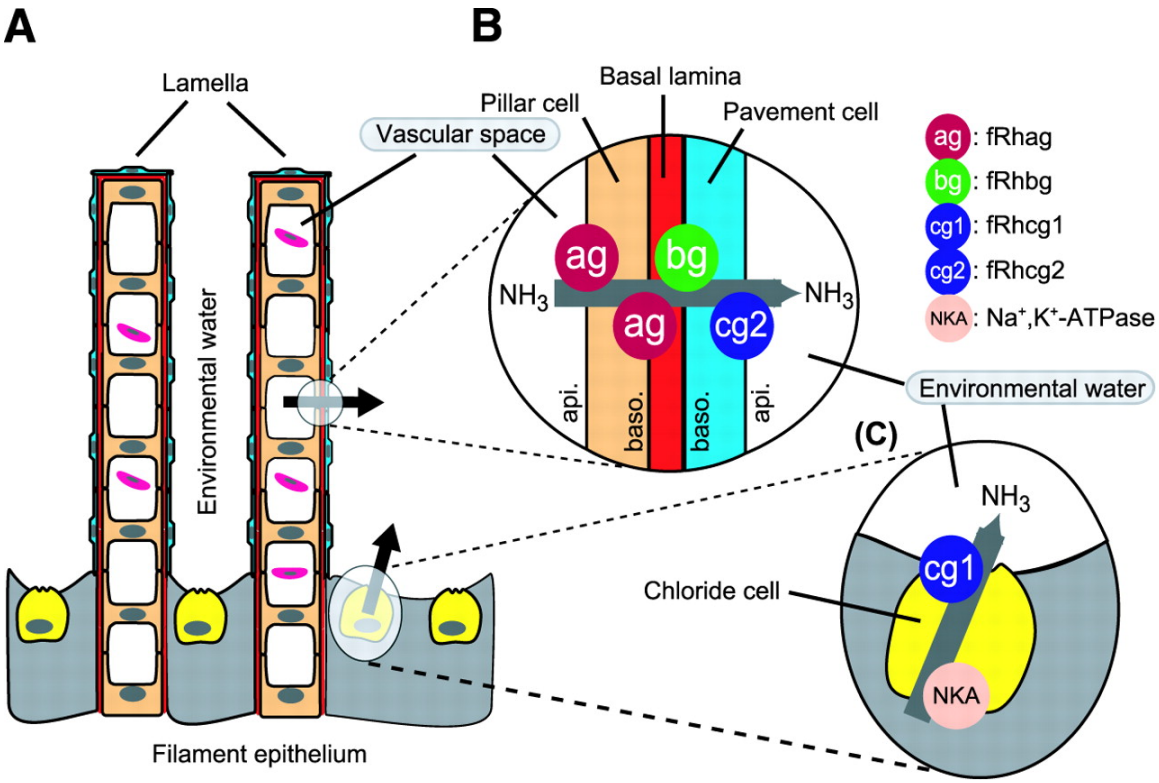
The hagfishes possess the longest known evolutionary history of the extant craniates and are likely to offer exclusive insight into vertebrate origins. The Atlantic hagfish is physiologically adapted to spend much of its life burrowed in ocean-floor substrate and marine carcasses, where high environmental ammonia and unfavorable conditions for ammonia excretion are likely encountered. The current study presents evidence that suggests Atlantic hagfish are capable of reducing experimentally elevated plasma ammonia concentrations and eventually eliminating the ammonia load within 8 hours. Following the injection of ammonia, elevated plasma ammonia concentrations paralleled elevated ammonia excretion rates and coincided with the initial upregulation of Rhcg and Rhbg in the gill and Rhcg in the skin suggesting that the transcriptional regulation of Rh glycoproteins may respond, in part, to elevated plasma ammonia. The subsequent translation of upregulated Rhcg mRNA into protein provides evidence that suggests Rh glycoproteins are involved in the regulation of ammonia excretion in Atlantic hagfish.

Table 1. Atlantic hagfish specific primers used for relative quantification of Rhcg and Rhcg mRNA expression by qRT-PCR.

Primers	Tm	Sequence	Product size
18S F2	58.0	5’ GCTCTTGGATGAGTGTCCGTTG 3’	250 bp
18S R2	54.8	5’ TTCTTGGCAAATGCTTTCGC 3’	
Rhcg F1	54.6	5’ GGTGGCACTATTGTCGGTAT 3’	91 bp
Rhcg R1	54.2	5’ CCTCCCAATATGCTCTGTCTT 3’	
Rhbg F1	55.0	5’ CGATAGGAGGTGGCATGATTAC 3’	77 bp
Rhbg R1	54.5	5’ GAAGCAATCAACATCAGAAGCC 3’	

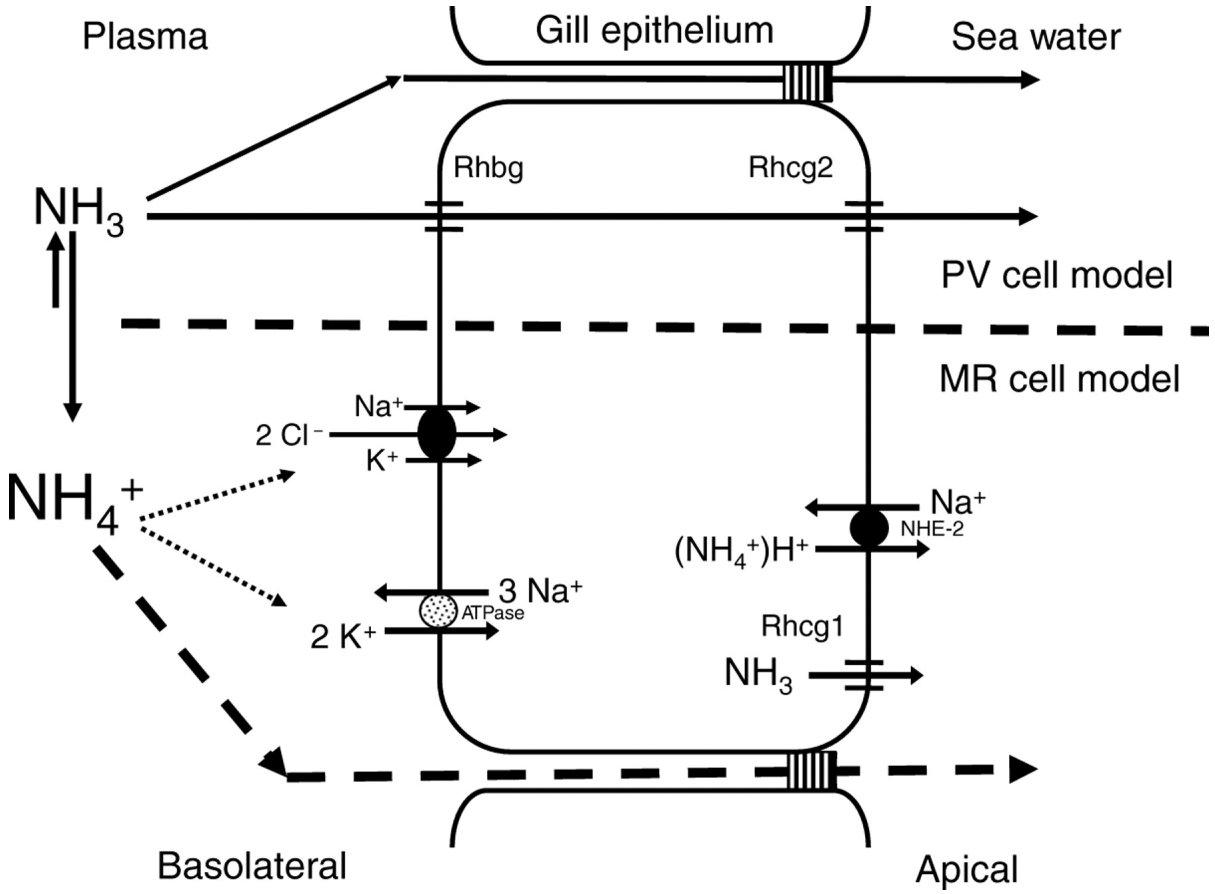
Table 2. Sample size and individual mass of Atlantic hagfish injected with seawater sham or ammonia.

		Mass of individuals (g)			
Injection	**N**	**Minimum**	**Maximum**	**Mean**	**SEM**
Sham	38	40	113	76.1	2.7
Ammonia	38	47	115	79.4	2.6



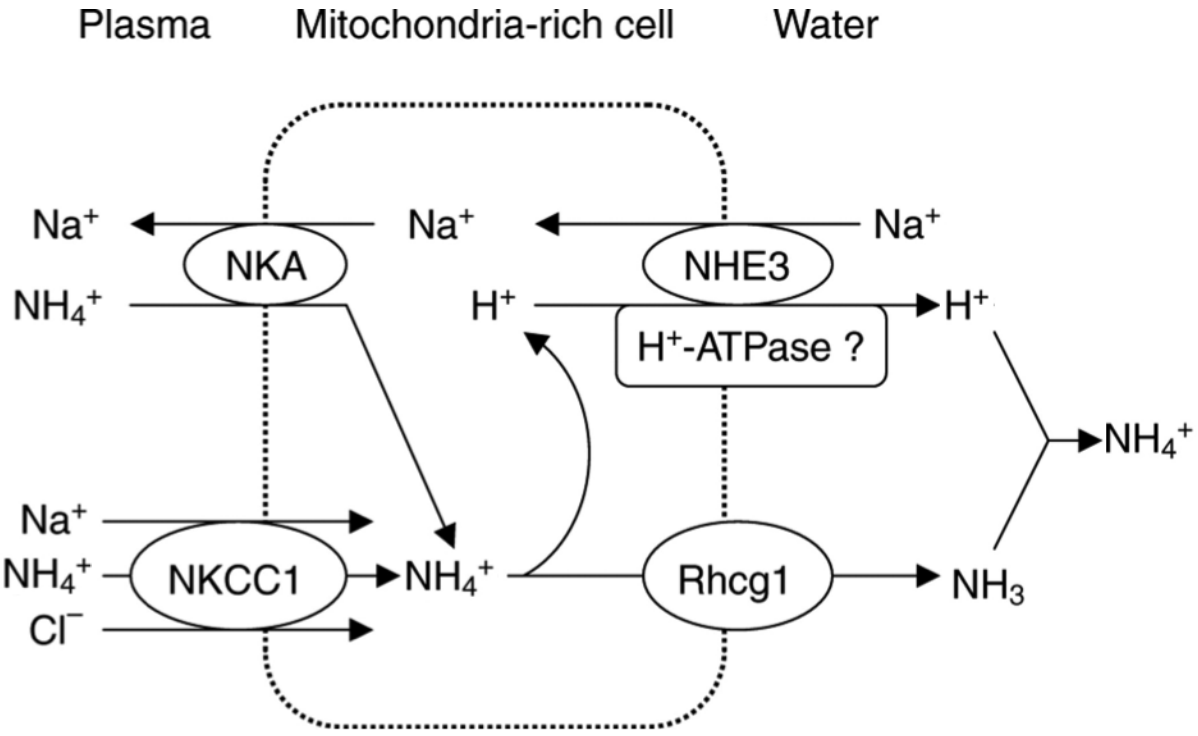
(Nakada et al., 2007a)

Fig. 1. Conceptual model of branchial ammonia excretion mediated by Rh glycoproteins in pufferfish. A) Structural depiction of gill lamellae and filament with MRC or chloride cells. B) Lamellar model for the facilitated transcellular diffusion of ammonia from the vascular space via Rhag across the pillar cell to the basal lamina, then across the pavement cell via Rhbg and Rhcg2 to environmental water. C) MRC or chloride cell model of ammonia transport from the filament epithelium to environmental water via a coupling of Rhcg1 and Na+, K+-ATPase (Nakada et al., 2007a).



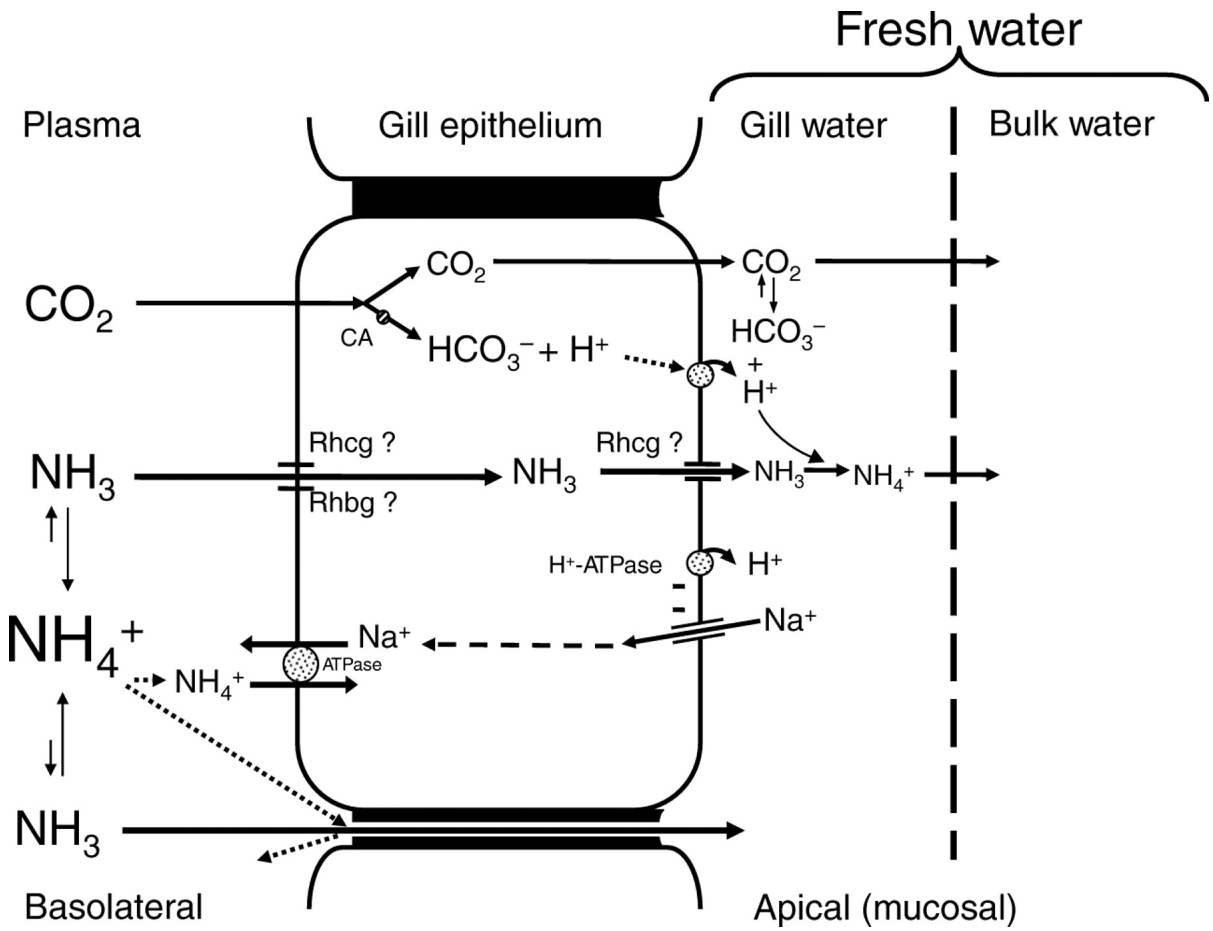
(Weihrauch et al., 2009)

Fig. 2. Proposed model of branchial ammonia excretion in saltwater teleosts. Really, two models are presented, one for pavement (PV) cells and one for MR cells. For pavement cells, the model involves basolaterally located Rhbg and apically located Rhcg2, but adds that significant diffusion of NH_3 and NH_4^+ likely occurs across the shallow paracellular junction of gill epithelial cells in marine fishes. The proposed MRC model is primarily focused on the transport of NH_4^+ into the MRCs via basolaterally located NKA and NKCC and excreted as NH_4^+ substituted for H^+ as the substrate of apically located Na^+/H^+-exchanger (NHE-2) or as NH_3 by Rhcg1 (Weihrauch et al., 2009)



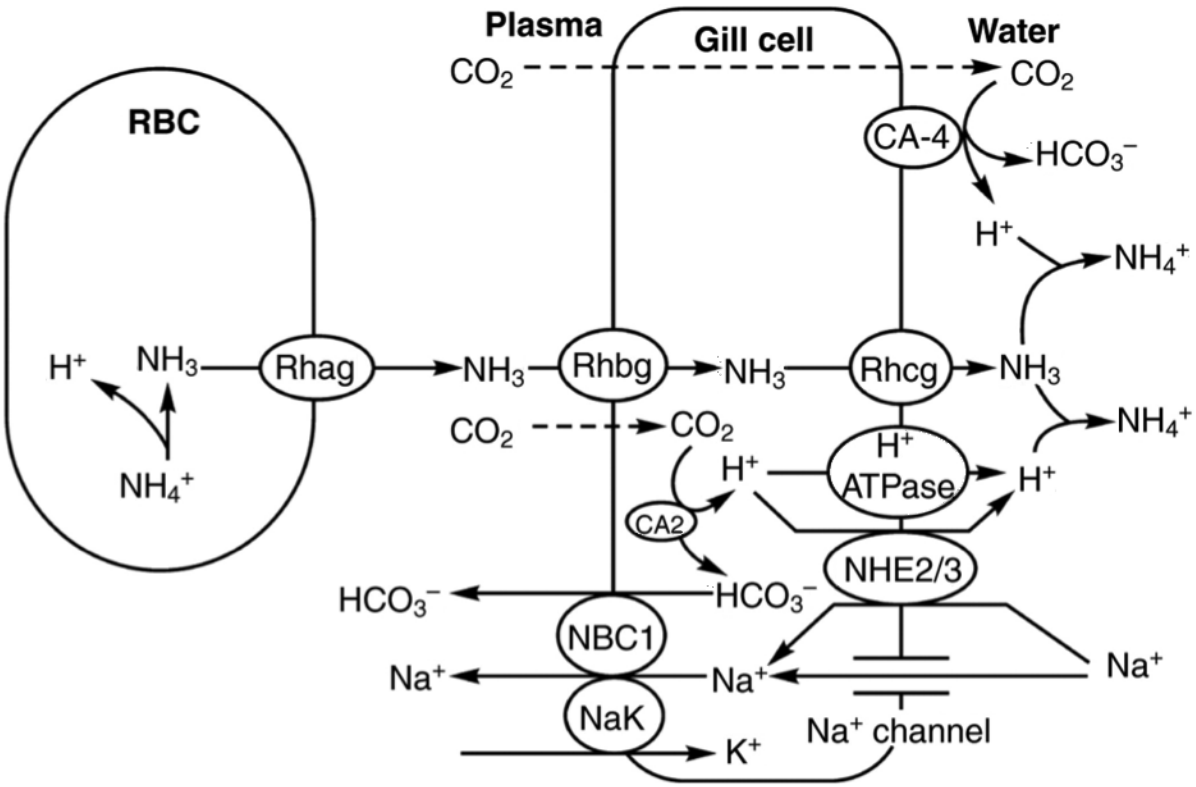
(Modified from Nawata et al., 2010)

Fig. 3. Proposed model of Rhcg1-mediated active ammonia excretion in MRCs. Nawata et al. (2010) speculated that during HEA, as the NH_3 gradient is unfavorable for passive diffusion, ammonia excretion is likely driven by active mechanisms (basolaterally located NKA and NKCC1, as well as apical H^+-ATPase and NHE-3) available in the MRCs (Fig. 3). In this model, it is assumed that most NH_4^+ is deprotonated prior to excretion via apical Rhcg1. The backflow of NH_3 is further prevented by combination with H^+ excreted by H^+-ATPase or NHE-3.



(Weihrauch et al, 2009).

Fig. 4. Proposed model of branchial ammonia excretion in freshwater teleosts. Weihrauch et al. (2009) propose a model utilizing an unspecified combination of Rh glycoproteins in the transcellular movement of ammonia across the gill epithelium and requires acidification of the gill boundary layer by H^+ excretion and CO_2 hydration to maintain a favorable ammonia diffusion gradient.



(Modified from Wright and Wood, 2009).

Fig. 5. Proposed model of branchial ammonia excretion in freshwater teleosts, including transport from the red blood cells. In this model Rhag located on the red blood cells (RBC) are utilized to transport ammonia across the RBC membrane into the plasma and basolaterally located Rhbg facilitates transport into the gill epithelium. Apical Rhcg (variant not specified) facilitates the outward diffusion of NH_3 into the gill boundary layer water, where acid trapping (combination with H^+ provided by NHE2/3, HAT, or CO_2 hydration to form NH_4^+) is utilized to maintain a favorable diffusion gradient.

5’-
GGGGGTTGCCACAATATACACCTGCTGTATTACCGGAGTCAATATTGTGTGGTGCTGAC
GAGTCCTTGCGGAGCCATGGGGTCATTTCCAAACCTTCGCATCCGACTTCCACTCGTAGC
TTTCCTCCTTGAGCTTCTCATGATTATTCTCTTTGGCGTATTTGTGAAGTACGATGATG
AATCCGATGCTTCAAAACCTGATGATCTGACGGGAAAATCTACTTTCCTTTACCGATAT
CCCAGTTTCCAGGATGTCCATGTCATGGTTTTCATGGGATTTGGGTTCCTCATGACGTT
CCTGCAGCGTTACGGCTTTAGCGCGGTTGGTTTCAACTTCCTCGTCGCTTCTTTCTCCCT
TCAGTGGGCGACGCTCATGCAGGGCTGGTTTCATCACTTCCAGGATGGCAAGATCCTTG
TCGGAGTGGAAAGCCTCATCAATGCGGATTTTTGCGCAGCATCTATGCTCATTGCCTTT
GGGGCCGTGTTGGGGAGGACCAGGCCCGTACAGCTCCTTATTATGGCCTTTTTCCAAGT
GACGTTATTTTCAGTCAATGAGTATATCCTTCTCAACCTGCTTGAGGTAATCGATGCAC
GTGGATCTATGACCATCCACTGCTTTGGTGGATTTTTTGGTCTCGCGGTTTCTCGAGTC
CTATACCGTCCAGGCCTCAAGGAGCCCCACCGAAAGGCATCTTCAGTCTATCACTCTGAT
TTATTTGCTATGATTGGTACTCTGTTCCTATGGATTTACTGGCCGAGCTTTAATTCTGC
AATTTCAGAAAAAGGTGTAAATCAGACTCGAGCAGTAATCAATACATACTACACGCTGG
CATCGTGTACTGTAACTACGTGTATCCTATCCAGCTTGGTTGACAAGAGCGGCCGAATC
AACATGGTCCATTTGCAGAGTTCGACGTTGGCCGGAGCAGTTGCGGTTGGAACGGCAGC
AGAAATGATGCTCACTCCGTACGGGTCCCTTATCGTGGGGTTAATTCTGGGAACGCTCA
GCACACTTGGATACACCTTCATCACGCCTGCCCTGGAAAAATACCTCCATGTTCAAGACA
CCTGTGGCATTCATAACCTGCATGCCCTTCCTGGCTTCTGCGGAGGTATCATTGGCGCAA
TTACAGCTGCTGCTGCTTCAGAAGCGACTTATGGTTCAAGACTCCACCACACCTTTCCAT
CTCTGGATAAACATCCGGGTCGGTCTTTGGGTGGTTTTCAGATGGCAGGAGCCGTTGTC
TCCCTCGGCATGGGCTTGGTTGGTGGCACTATTGTCGGTATTATTCTTAAATTCCCTAT
CTGGGGGGCCACGACTGATGAAGATTGTTATGAAGACAGAGCATATTGGGAGGTTCCAG
AGGAGGAGGTAACATACATTCATACAGTAACACGAAATGAACACGTGAAAGAGGAAAA
CACCGTGTATGAAATGGAGCCACAAAGTCCAGCTGACTAA
-3’

Fig. 6. Atlantic hagfish Rhcg cDNA nucleotide sequence from mRNA (Edwards, S. L. and Walsh, P. J., GenBank: GU733440).

5’-
ATGGCGATCTACCACACCAAAATGAACCCCAAGCTACCAGTGGTGGCACTGGTGCTTGA
GGTTGTGATGCTTATCCTGTTTGGCATATTTGTACGATATGACCACGATGCCGATGCAA
AGGATCATCATAATAACACCAAAGCACAGAAAAACGAGTTCTACTTCAGATACGCAAGC
TTTCAAGATGTACACGTGATGATTTTCATTGGTTTTGGCTTCCTCATGACTTTCCTTAA
GCGCTACAGTTTCAGCAGCGTGGGTTTCAACTTCTTAATCGCAGCATTTGGTCTGCAAT
GGGCAGTGCTACTGCAAGGATGGTTGCACCACTTCGACTCTAGCACTATGAAAATCCAC
ATTGGCATGGAGGGGATGATAAATGCCGATTTCTGTACAGCAGCCGTTCTCATCAGCTT
TGGTGCCGTGTTGGGTAAAACAAGTCCTTTACAACTGTTGGTGATGACAGTACTAGAAG
AGGTTTTCTTTGCTCTGAATGAACACATCGCCATAGGTATTTTACAGGTGAATGATGCT
GGTGGATCAATGGTCATACACCTGTTTGGAGCCTATTTTGGTCTTGCTGTCTCCAGAGT
AATGTTCCGTGATGGCTTGCACGGAGAGACGCATGAAAAAGAGGGCTCTGTTTACCACT
CGGACGTCTTTGCCATGATTGGCACCATCTTCCTGTGGATGTTCTGGCCAAGTTTTAAT
TCTGCCATCGCTGGTCATGGAGATGATCAAATGCGCGCTGCGATGAATACATATTTCTC
ACTCGCTGCCAGCCTTGTGGCCACTTTTGCAATTTCGAGTTTGACAAGTCGAGAGGGAA
AGTTGGACATGGTTCACATTCAGAATGCCTCACTAGCTGGAGGCGTGGCTGTGGGGACG
TGTGCCGACATGATGATAAACCCGTATGGAGCTATCATGATTGGCTTTTGCAGCGGTAT
AGTTTCTACCTTGGGCTTCAAGTATCTTGCGCCGCTTTTGGAATCAAAGTTGAAAGTTC
ACGACACATGTGGCGTTCACAACTTGCACGGAATGCCAGGAATTCTGGGAGGACTTTCT
GGAGCCATTGCTGCGGCCTTTGCTAGTGAAGAAGTCTATGGACTGAGCTTGTACTTGAC
CTTTCCAACATTAGTGCCAGAAGACAAAAACACTCCTCTGTTCAAACGTCTTCACGAAT
GGAGCGATGAAATTGAACCTGGAAAGGGACGCTCGCCCATGGTGCAGGGGGGGTTCCAG
GCTCTGCAGGTCCTCATCTCCTTGGTCATGGCGATAGGAGGTGGCATGATTACAGGTTT
CTTCCTCAAACATACTTGGTGGGGGCAGGCTTCTGATGTTGATTGCTTCGAGGACAGCA
TGTACTGGGAGGTGCCTGAAGCTGAGGGGTTTACAGAGGTCAAAACTGATGGAGAGCA
TCCCTTGGATACGCAGGACGAGCTCTAA
-3’

Fig. 7. Atlantic hagfish Rhbg cDNA nucleotide sequence from mRNA (Edwards, S. L. and Walsh, P. J., GenBank: GU733441).

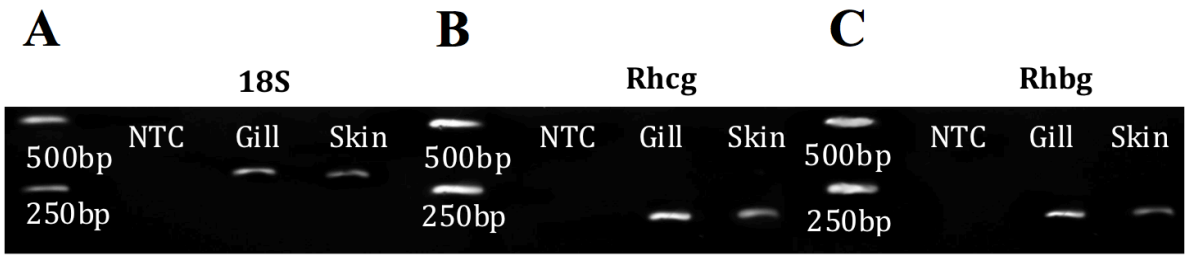


Fig. 8. RT-PCR products separated by 1% Ethidium bromide gel electrophoresis. (A) hagfish 18S, (B) hagfish Rhcg, and (C) hagfish Rhbg were amplified from reverse transcribed mRNA isolated from gill and skin (see Table 1 for primer information). Molecular grade water was substituted for template cDNA in no template control (NTC) reactions.

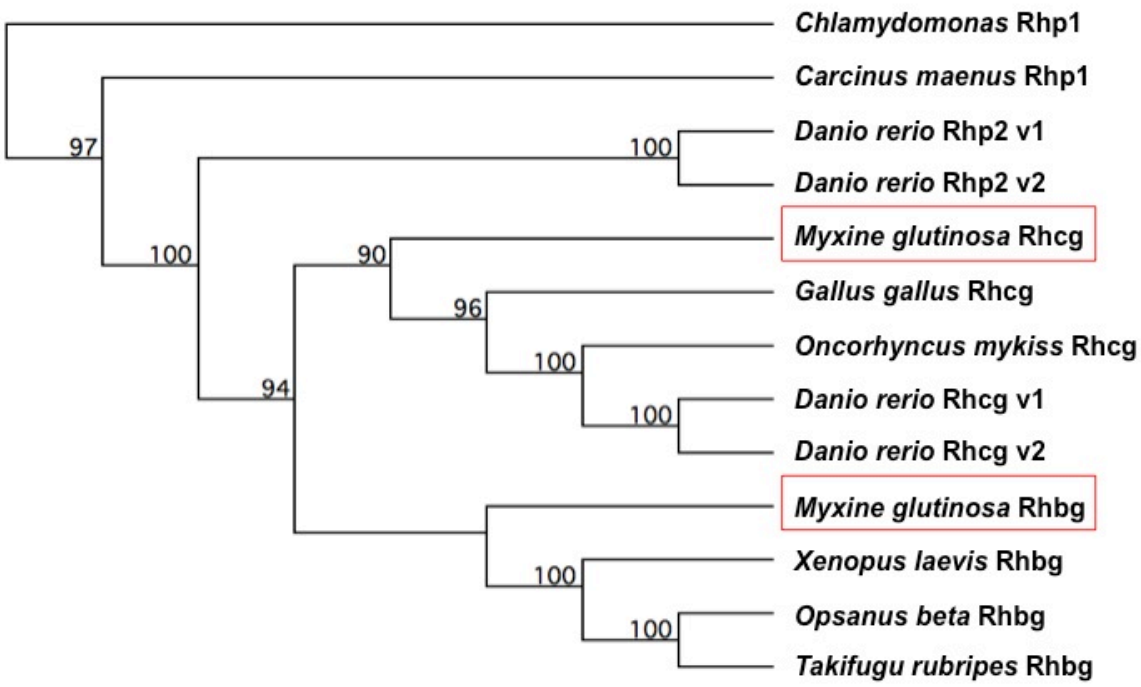


Fig. 9. Phylogenetic reconstruction of Rh glycoproteins. The tree is rooted to *Chlamydomonas* Rhp1. Clades formed for Rhcg, Rhbg, Rhp2 and Rhp1. *Myxine glutinosa* Rhcg and *Myxine glutinosa* Rhbg were ancestral to the respective isoforms of higher vertebrates, but were not ancestral to the *Danio rerio* Rhp2.

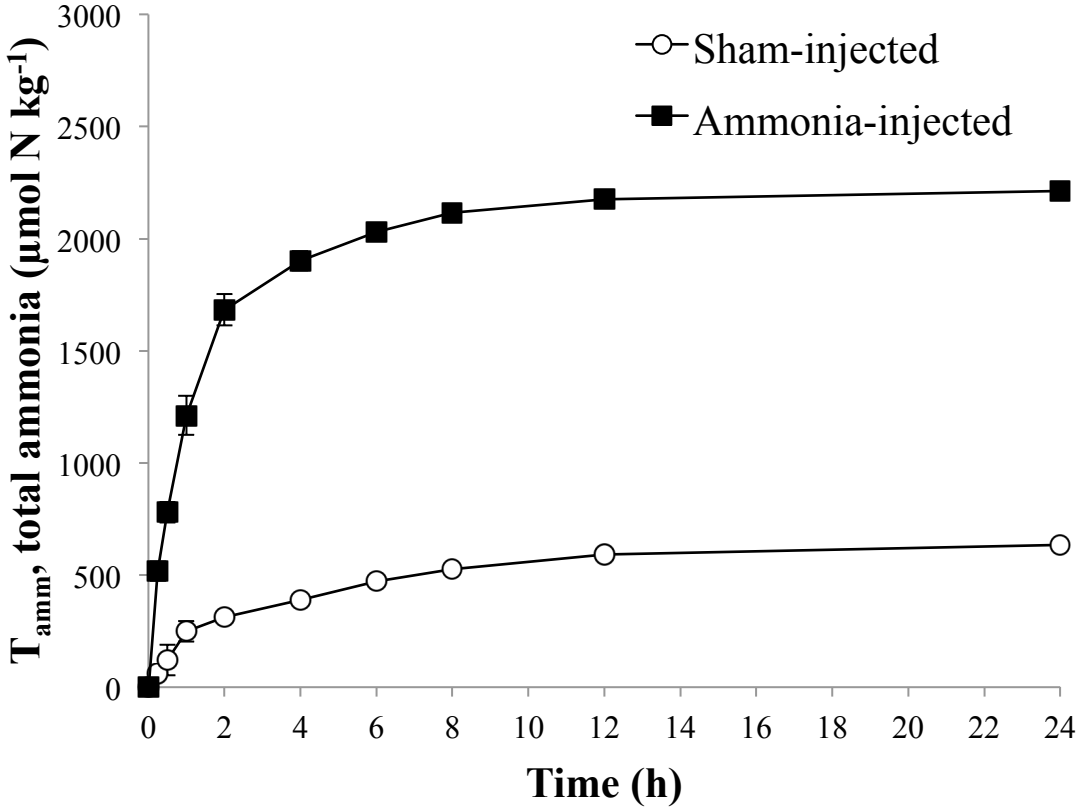


Fig. 10. Cumulative total ammonia in the environmental water. Total ammonia concentrations of ammonia-injected hagfish remained elevated (P>0.05) above sham-injected hagfish at all time points following injection (zero hours).

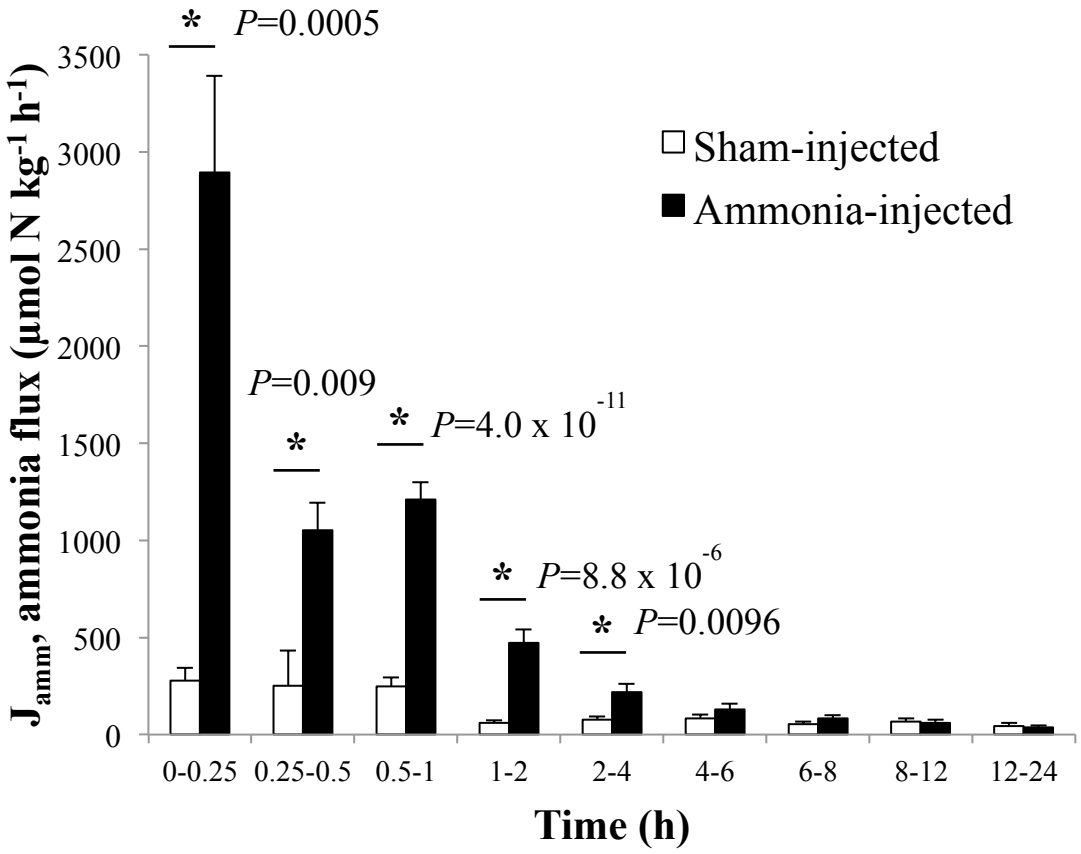


Fig. 11. Net flux of total ammonia in environmental water. Net flux rates were calculated from changes in cumulative total ammonia concentrations between time-points. The highest rate of excretion was observed during the 0.25 hour following injection of 100 mM NH_4Cl. Ammonia excretion remained elevated above sham-injected levels until 6 hours post-injection. * denotes a statistically significant difference (P>0.05) between treatments at a time-point.

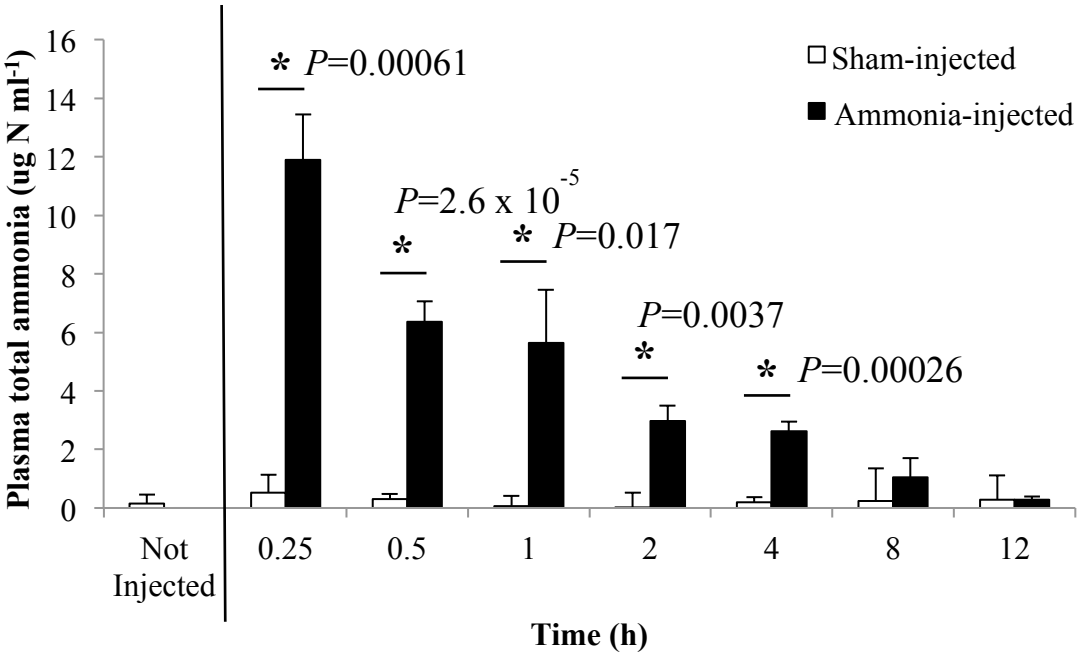


Fig. 12. Plasma total ammonia profile. Plasma ammonia concentrations were significantly elevated in ammonia-injected hagfish through 4 hours post-injection. *N* of 5 individuals per treatment at each time point, except for 12 hours (*N* of 3 individuals per treatment). * denotes a statistically significant difference ($P>0.05$) between treatments at a time-point.

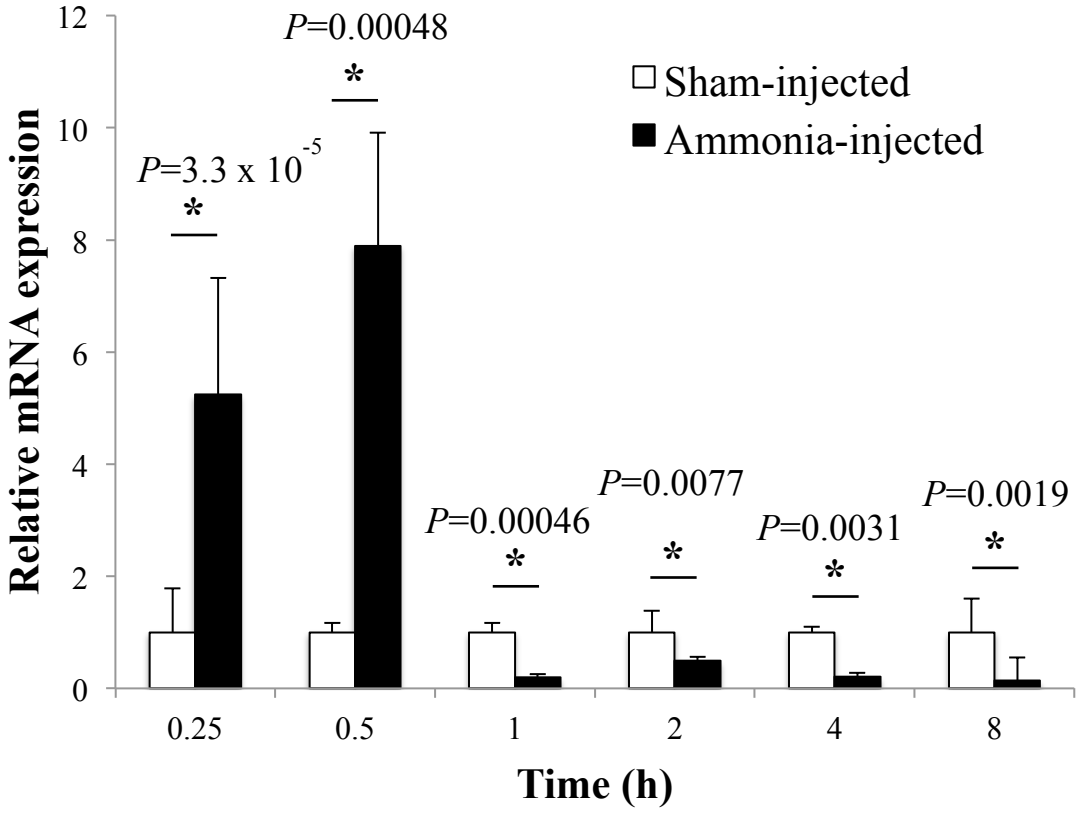


Fig. 13. Rhcg mRNA expression in gill tissue. Expression of Rhcg mRNA in the ammonia-injected group was upregulated at 0.25 and 0.5 hours post-injection and downregulated at all subsequent time-points relative to the sham-injected group. *N* of 5 individuals per treatment at each time point. * denotes a statistically significant difference ($P>0.05$) between treatments at a time-point.

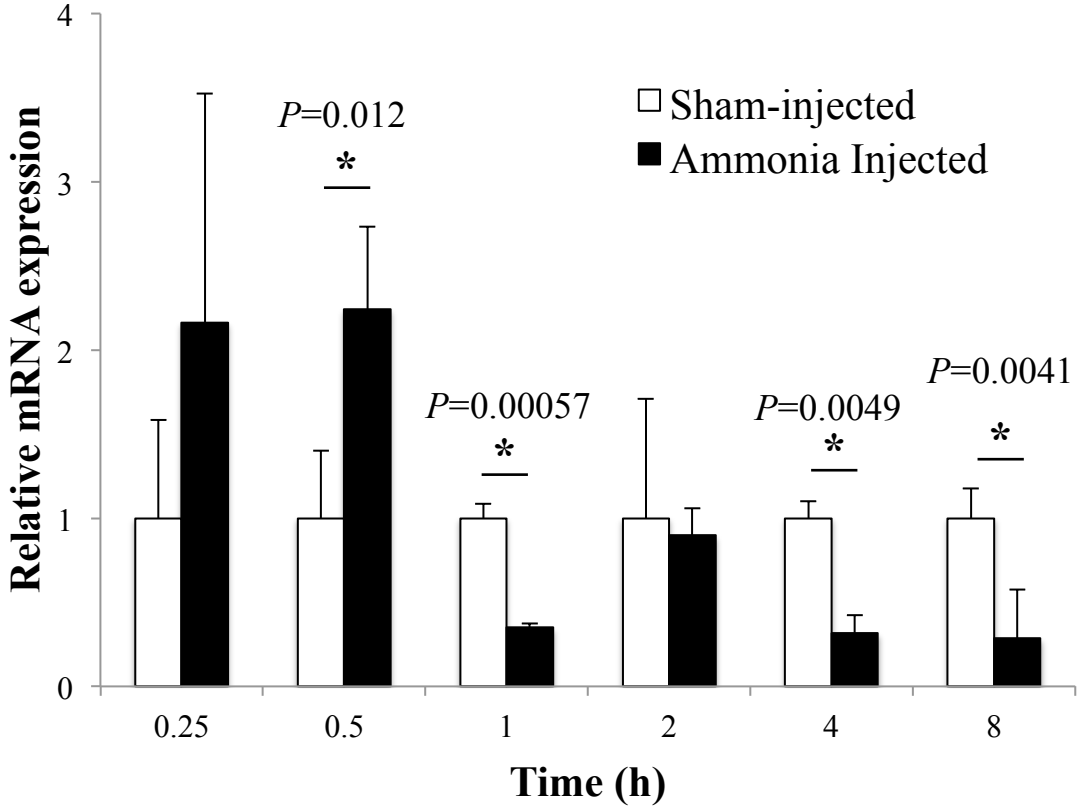


Fig. 14. Rhbg mRNA expression in gill tissue. Expression of Rhbg mRNA in the ammonia-injected group was upregulated at 0.5 hours post-injection, but downregulated at 1, 4, and 8 hours post-injection relative to the sham-injected group. *N* of 5 individuals per treatment at each time point. * denotes a statistically significant difference ($P>0.05$) between treatments at a time-point.

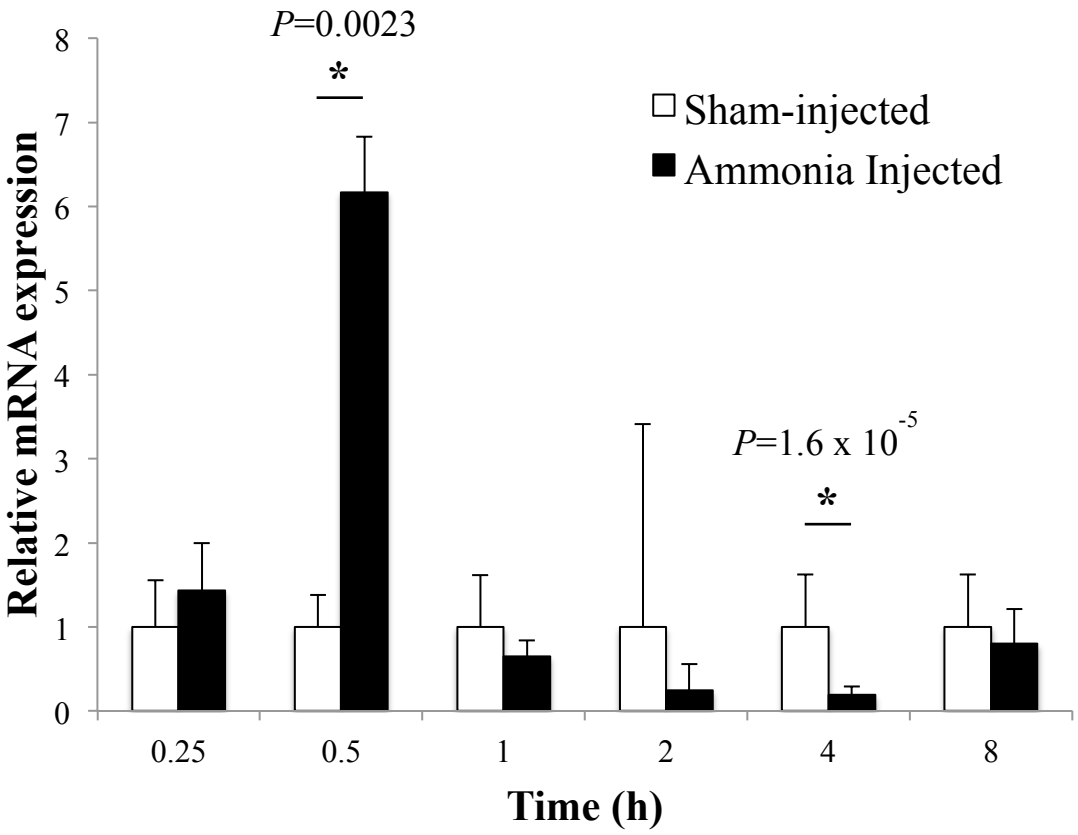


Fig. 15. Rhcg mRNA expression in skin tissue. Expression of Rhcg mRNA in the ammonia-injected group was upregulated at 0.5 hours post-injection, but downregulated at 4 hours post-injection relative to the sham-injected group. *N* of 5 individuals per treatment at each time point. * denotes a statistically significant difference ($P>0.05$) between treatments at a time-point.

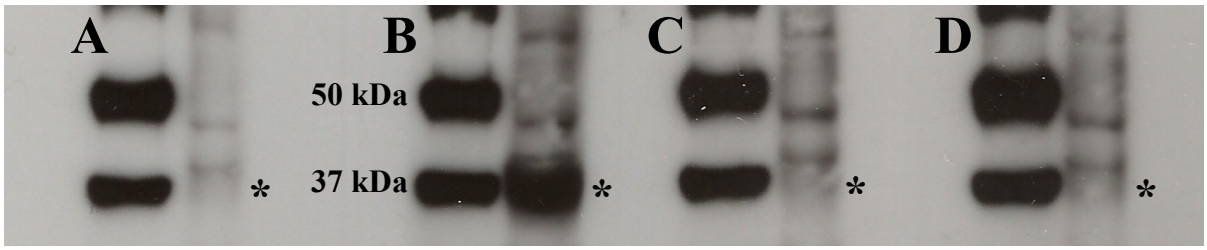
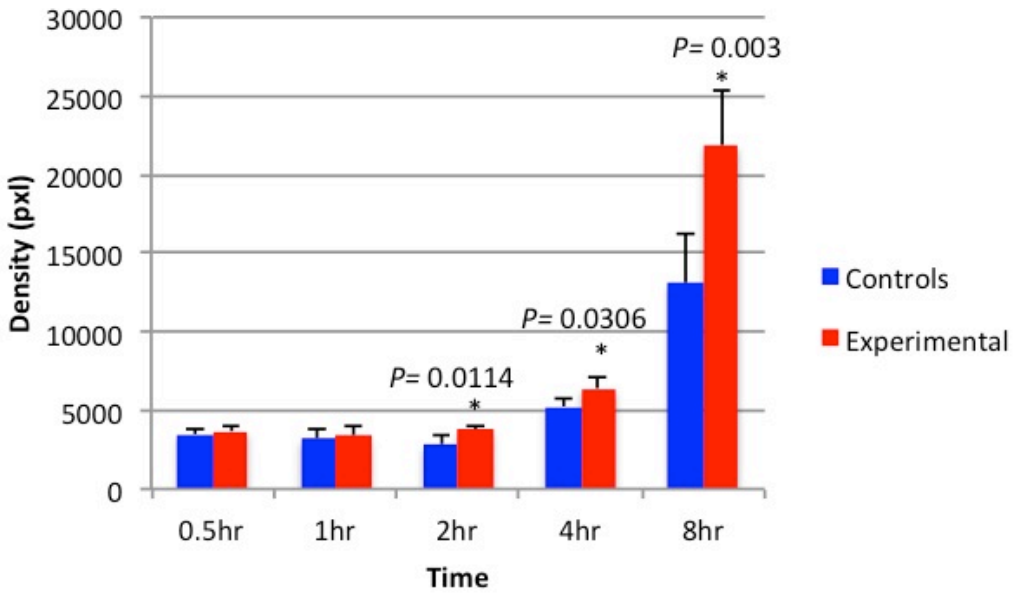
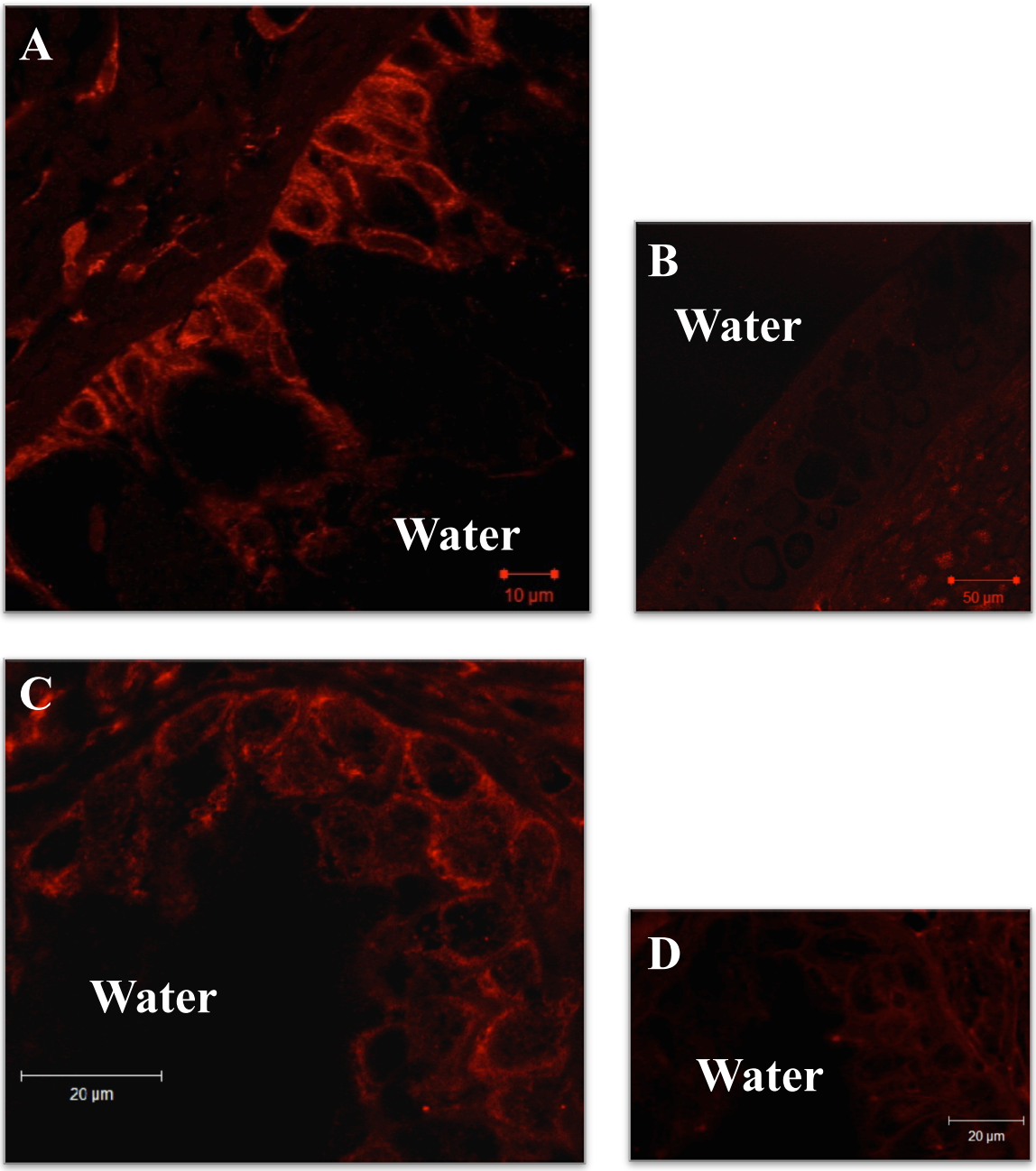


Fig. 16. Immunoblot using homologous hagfish Rhcg antibody. Western blot of Atlantic hagfish gill proteins separated by SDS-PAGE. The left lane of each pair contains molecular weight markers. A) Negative control. B) Positive control, 1:5,000 dilution of Rhcg antibody. C) Rhcg antibody preabsorbed 1:10 with purified Rhcg peptide, 1:5,000 dilution. D) Rhcg antibody preabsorbed 1:20 with purified Rhcg peptide, 1:5,000 dilution. * indicates the location of the immunoreactive in the positive control and the absence of the same band in the negative control and preabsorbed immunoblots.



Edwards (unpublished)

Fig. 17. Quantification of Atlantic hagfish Rhcg protein expression in gill tissue (Edwards, unpublished). *N* of 5 individuals per treatment at each time point. * denotes a statistically significant difference (*P*>0.05) between treatments at a time-point.



(Pray, 2013, unpublished Honor's thesis)

Fig. 18. Immunolocalization of hagfish Rhcg in (A) the gill and (C) skin of Atlantic hagfish. Note the immunoreactive staining associated with the epithelium closest to the blood margin in (A) and (D). Respective negative controls shown in B and D (Pray, 2012 unpublished thesis).

References

Bartels, H. (1988). Intercellular junctions in the gill epithelium of the Atlantic hagfish, *Myxine glutinosa*. *Cell Tissue Res.* **254**, 573–583.

Braun, M. H. and Perry, S. F. (2010). Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii*: Evidence for the involvement of Rh and UT proteins. *Comp. Biochem. Physiol.* **157A**, 405-415.

Brauner, C. J. and Baker, D. W. (2009). Patterns of acid–base regulation during exposure to hypercarbia in fishes. In *Cardio-Respiratory Control in Vertebrates* (ed. Glass, M. L. and Wood, S. C.), pp. 43–63. Springer Berlin Heidelberg.

Cherif-Zahar, B., Durand, A., Schmidt, I., Hamdaoui, N., Matic, I., Merrick, M., and Matassi, G. (2007). Evolution and functional characterization of the RH50 gene from the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *J. Bacteriol.* **189**, 9090–9100.

Choe, K. P., Edwards, S., Morrison-Shetlar, a I., Toop, T. and Claiborne, J. B. (1999). Immunolocalization of Na^+/K^+-ATPase in mitochondrion-rich cells of the Atlantic hagfish (*Myxine glutinosa*) gill. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **124**, 161–8.

Currie, S. and Edwards, S. L. (2010). The curious case of the chemical composition of hagfish tissues-50 years on. *Comp Biochem. Physiol.* **157A**, 111-115.

Cooper, A. J. and Plum, F. (1987). Biochemistry and physiology of brain ammonia. *Physiol. Rev.* **67**, 440-519.

Edwards, S. L., Claiborne, J. B., Morrison-Shetlar, A. L. and Toop, T. (2001). Expression of Na^+/ H^+ exchanger mRNA in the gills of the Atlantic hagfish (*Myxine glutinosa*) in response to metabolic acidosis. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **130**, 81–91.

Elger, M. (1987). The branchial circulation and the gill epithelia in the Atlantic hagfish, *Myxine glutinosa* L. *Anat. Embryol. (Berl).* **175**, 489–504.

Escriva, H., Manzon, L., Youson, J. H. and Laudet, V. (2002). Analysis of Lamprey and Hagfish genes reveals a complex history of gene duplications during early vertebrate evolution. *Mol. Biol. Evol.* **19**, 1440-1450.

Evans, D. H. (1984). Gill Na^+/H^+ and Cl^-/HCO_3^- exchange systems evolved before the vertebrates entered fresh water. *J. Exp. Biol.* **113**, 465–469.

Evans, D. H. (2011). Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. *Acta Physiol.* **202**, 349–59.

Evans, D. H., Piermarini, P., and Choe, K. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Forey, P., and Janvier, P. (1993). Agnathans and the origin of jawed vertebrates. *Nature* **361**, 129-134.

Glover, C., Bucking, C. and Wood, C. (2013). The skin of fish as a transport epithelium: a review. *J. Comp. Physiol. B* **183**, 877–891.

Good, D. W., Knepper, M. A. and Burg, M. B. (1984). Ammonia and bicarbonate transport by thick ascending limb of rat kidney. *Am. J. Physiol. Renal Physiol.* **247**, F35–F44.

Greenaway, P. and Nakamura, T. (1991). Nitrogenous excretion in two terrestrial crabs (*Gecarcoidea natalis* and *Geograpsus grayi*). *Physiological Zoology* **64**, 767–786.

Han, K.-H., Kim, H.-Y. and Weiner, I. D. (2009). Expression of Rh glycoproteins in the mammalian kidney. *Electrolyte & Blood Pressure* **7**, 14–9.

Heimberg, A. M., Cowper-Sal-lari, R., Sémon, M., Donoghue, P. C. J. and Peterson, K. J. (2010). microRNAs reveal the interrelationships of hagfish, lampreys, and gnathostomes and the nature of the ancestral vertebrate. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 19379–83.

Huang, C.H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15512-15517.

Hung, C. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J. Exp. Biol.* **210**, 2419-2429.

Hwang, P.-P., Lee, T.-H. and Lin, L.-Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R28-R47.

Ip, Y. K. and Chew, S. F. (2010). Ammonia production, excretion, toxicity, and defense in fish: a review. *Front. Physiol.* **1**, 134.

Ip, Y. K., Chew, S. F. and Randall, D. J. (2001). Ammonia toxicity, tolerance, and excretion. In *Nitrogen Excretion* (ed. P. A. Wright,and P. M. Anderson), pp. 109–148. New York, NY: New Academic Press.

Janvier, P. (1981). The phylogeny of the Craniata, with particular reference to the significance of fossil "agnathans." *J. Vert. Paleontol.* **1**, 121–159.

Kikeri, D., Sun, A., Zeidel, M. L. and Hebert, S. C. (1989). Cell membranes impermeable to NH_3. *Nature* **339**, 478–480.

Krogh, A. (1939). *Osmotic regulation in aquatic animals*. Cambridge: Cambridge University Press.

Kumai, Y. and Perry, S. F. (2011). Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **031**, R1517-R1528.

Lesser, M., Martini, F. and Heiser, J. B. (1996). Ecology of the hagfish, *Myxine glutinosa* L. in the Gulf of Maine: I. Metabolic rates and energetics. *J. Exp. Marine Biol. Ecol.* **208**, 215-225.

Levine, P. and Stetson, R.E. (1939). An unusual case of intra-group agglutination. *J. Am. Med. Assoc.* **113**, 126-127.

Liu, S.-T., Tsung, L., Horng, J.-L. and Lin, L.-Y. (2013). Proton-facilitated ammonia excretion by ionocytes of medaka (*Oryzias latipes*) acclimated to seawater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **305**, R242–R251.

Ludewig, U. (2004). Electroneutral ammonium transport by basolateral rhesus B glycoprotein. *J. Physiol.* **559**, 751-759.

Mallatt, J., Conley, D. M. and Ridgway, R. L. (1987). Why do hagfish have gill "chloride cells" when they need not regulate plasma NaCl concentration? *Can. J. Zool.* **65**, 1956–1965.

Maetz, J. and García Romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus* : II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.* **47**, 1209–1227.

Marini, A. M., Urrestarazu, A., Beauwens, R. and André, B. (1997). The Rh (Rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem. Sci*. **22**, 460-461.

Marini, A. M., Matassi, G., Raynal, V., Andre, B., Cartron, J. P. and Cherif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat. Genet*. **26**, 341–344.

Martini, F., Lesser, M. and Heiser, J. B. (1997). Ecology of the hagfish, *Myxine glutinosa* L., in the Gulf of Maine: II. Potential impact on benthic communities and commercial fisheries. *J. Exp. Marine Biol. Ecol.* **214**, 97-106.

Matassi, G., Cherif-Zahar, B., Pesole, G., Raynal, V. and Cartron, J.P. (1999). The members of the RH gene family (RH50 and RH30) followed different evolutionary pathways. *J. Mol. Evol.* **48**, 151-159.

McDonald, G., Cavdek, V., Calvert, L. and Milligan, C. L. (1991). Acid-base regulation in the Atlantic hagfish *Myxine glutinosa*. *J. Exp. Biol.* **161**, 201–215.

McDonald, T. R., Dietrich, F. S. and Lutzoni, F. (2012). Multiple horizontal gene transfers of ammonium transporters/ammonia permeases from prokaryotes to eukaryotes: toward a new functional and evolutionary classification. *Mol. Biol. Evol.* **29**, 51-60.

Monfort, P., Cauli, O., Montoliu, C., Rodrigo, R., Llansola, M., Piedrafita, B., el Mlili, N., Boix, J., Agustí, A. and Felipo, V. (2009). Mechanisms of cognitive alterations in hyperammonemia and hepatic encephalopathy: Therapeutical implications. *Neurochem. Int.* **55**, 106–112.

Nakada, T., Westhoff, C.M., Kato, A. and Hirose, S. (2007a). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J.* **21**, 1067-1074.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007b). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **293**, R1743-1753.

Nakada, T., Westhoff, C. M., Yamaguchi, Y., Hyodo, S., Li, X. J., Muro, T., Kato, A., Nakamura, N. and Hirose, S. (2010). Rhesus glycoprotein P2 (Rhp2) is a novel member of the Rh family of ammonia transporters highly expressed in shark kidney. *J. Biol. Chem.* **285**, 2653-2664.

Nawata, C. M. and Wood, C. M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* **211**, 3226-3236.

Nawata, C. M., Hung, C. C., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics* **31**, 463-474.

Nawata, C. M., Hirose, S., Nakada, T., Wood, C. M. and Kato, A. (2010). Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. *J. Exp. Biol.* **213**, 3150-3160.

Near, T. J. (2009). Conflict and resolution between phylogenies inferred from molecular and phenotypic data sets for hagfish, lampreys, and gnathostomes. *J. Exp. Zool. Part B, Mol. Dev. Evol.* **312**, 749–61.

Ota, K. G., Kuraku, S. and Kuratani, S. (2007). Hagfish embryology with reference to the evolution of the neural crest. *Nature* **446**, 672–675.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* **29**, e45.

Powell, M. L., Kavanaugh, S. I. and Sower, S. A. (2004). Seasonal concentrations of reproductive steroids in the gonads of the Atlantic hagfish*, Myxine glutinosa*. *J. Exp. Zool. Part A, Comp. Exp. Biol.,* **301**, 352–60.

Powell, M. L., Kavanaugh, S. I. and Sower, S. A. (2005). Current knowledge of hagfish reproduction: Implications for fisheries management. *Integr. Comp. Biol.* **45**, 158-165.

Pray, M. E. (2013). Localization of Rh glycoproteins in the Atlantic hagfish, *Myxine glutinosa*. Unpublished Senior Honor's Thesis, Appalachian State University, Boone, NC, USA.

Ripoche, P., Bertrand, O., Gane, P., Birkennmeier, C., Colin, Y. and Cartron, J. P. (2004). Human Rhesus-associated glycoprotein mediates facilitated transport of NH_3 into red blood cells. *Proc. Natl. Acad. Sci. USA* **101**, 17222-17227.

Rodela, T. M., Esbaugh, A. J., Weihrauch, D., Veauvy, C. M., McDonald, M. D., Gilmour, K. M. and Walsh, P. J. (2012). Revisiting the effects of crowding and feeding in the gulf toadfish, *Opsanus beta*: the role of Rhesus glycoproteins in nitrogen metabolism and excretion. *J. Exp. Biol.* **215**, 301-13.

Sardella, B. A., Baker, D. W. and Brauner, C. J. (2009). The effects of variable water salinity and ionic composition on the plasma status of the Pacific hagfish (*Eptatretus stoutii*). *J. Comp. Physiol. B* **179**, 721–728.

Sashaw, J., Nawata, M., Thompson, S., Wood, C. M. and Wright, P. A. (2010). Rhesus glycoprotein and urea transporter genes in rainbow trout embryos are upregulated in response to alkaline water (pH 9.7) but not elevated water ammonia. *Aquat. Toxicol.* **96**, 308-13.

Shelton, R. G. J. (1978). On the feeding of the hagfish *Myxine glutinosa* in the North Sea. *J. Mar. Biol. Assoc. U. K.* **58**, 81–86.

Shih, T.-H., Horng, J.-L., Hwang, P.-P. and Lin, L.-Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. **295**, C1625–C1632.

Shih, T.-H., Horng, J.-L., Lai, Y.-T. and Lin, L.-Y. (2013). Rhcg1 and Rhbg mediate ammonia excretion by ionocytes and keratinocytes in the skin of zebrafish larvae: H^+-ATPase-linked active ammonia excretion by ionocytes. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **304**, R1130–R1138.

Smith, H. W. (1929). The excretion of ammonia and urea by the gills of fish. *J. Biol. Chem.* **81**, 727–742.

Speeg, K. V and Campbell, J. W. (1968). Formation and volatilization of ammonia gas by terrestrial snails. *American Journal of Physiology* **214**, 1392–1402.

Strahan, R. (1963). The behaviour of myxinoids. *Acta Zool.* **44**, 73–102.

Takechi, M., Takeuchi, M., Ota, K. G., Nishimura, O., Mochii, M., Itomi, K., Adachi, N., Takahashi, M., Fujimoto, S., Tarui, H. et al. (2011). Overview of the transcriptome profiles edentified in hagfish, shark, and bichir: Current issues arising from some nonmodel vertebrate taxa. *J. Exp. Zool.* **316B**, 526-546.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.

Towle, D. W. and Holleland, T. (1987). Ammonium ion substitutes for K^+ in ATP-dependent Na^+ transport by basolateral membrane vesicles. **252**, R479–R489.

Tresguerres, M., Parks, S. K. and Goss, G. G. (2006). V-H^+-ATPase, Na^+/K^+-ATPase and NHE2 immunoreactivity in the gill epithelium of the Pacific hagfish (*Epatretus stoutii*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **145**, 312–321.

Tresguerres, M., Parks, S. K. and Goss, G. G. (2007). Recovery from blood alkalosis in the Pacific hagfish (*Eptatretus stoutii*): Involvement of gill V–H^+–ATPase and Na^+/K^+–ATPase. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **148**, 133–141.

Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2008). In vitro evidence for the involvement of Rh proteins and H^+ efflux in ammonia excretion across the gills of the freshwater rainbow trout. *Comp. Biochem. Physiol.* **150A**, S105-S105.

Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. *J. Exp. Biol.* **212**, 878-892.

Walsh, P. J. (1998). Nitrogen excretion and metabolism. In *The Physiology of Fishes,* 2nd edn. (ed. D. H. Evans), pp. 199–214. Boca Raton, FL: CRC Press.

Walsh, P. J., Wang, Y., Campbell, C. E., De Boeck, G. and Wood, C. M. (2001). Patterns of nitrogenous waste excretion and gill urea transporter mRNA expression in several species of marine fish. *Marine Biology* **139**, 839-844.

Walsh, P. J., Veauvy, C. M., McDonald, M. D., Pamenter, M. E., Buck, L. T. and Wilkie, M. P. (2007). Piscine insights into comparisons of anoxia tolerance, ammonia toxicity, stroke and hepatic encephalopathy. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **147**, 332–343.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39, 971-974.**

Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J. Exp. Biol.* **212**, 1716-1730.

Weisbart, M. and Idler, D. R. (1970). Re-examination of the presence of corticosteroids in two cyclostomes, the Atlantic hagfish (*Myxine glutinosa* L.) and the sea lamprey (*Petromyzon marinus* L.). *J. Endocrinol.* **46**, 29–43.

Westhoff, C. M. (2007). The structure and function of the Rh antigen complex. *Semin. Hematol.* **44**, 42-50.

Wieser, W., Schweizer, G. and Hartenstein, R. (1969). Patterns in the release of gaseous ammonia by terrestrial isopods. *Oecologia* **3**, 390–400.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J. Exp. Zool*. **293**, 284-301.

Wilkie, M. P. and Wang, Y. (1999). Nitrogenous waste excretion by the larvae of a phylogenetically ancient vertebrate: the sea lamprey (*Petromyzon marinus*). *Can. J. Zool.* **715**, 707–715.

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. *J. Exp. Biol.* **198**, 273–281.

Wright, P. A. (2007). Ionic, osmotic, and nitrogenous waste regulation. In Primitive Fishes, Fish Physiology Series, Vol. 26 (ed. D. J. McKenzie A. P. Farrell and C. J. Brauner), pp. 283-318. San Diego: Academic Press.

Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* **212**, 2303-12.

Wright, P. A. and Wood, C. M. (2012). Seven things fish know about ammonia and we don't. *Respir. Physiol. Neurobiol.* **184**, 231–240.

Wright, P. A., Randall, D. and Perry, S. (1989). Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J. Comp. Physiol. B* **158**, 627–635.

Vita

Justin M. Arnold became the second son born to parents Philip and Evelyn of Piney Creek, North Carolina in March of 1986. His family, especially his older brother, Jason, fostered a love for exploring the woods and rolling fields of Ashe and Alleghany counties. A notoriously inquisitive mind and penchant for tinkering naturally carried him to pursue studies in the biological sciences. He received his Bachelor of Science in Biology from Appalachian State University in May of 2008 following four years of dabbling in whatever research projects he could get his nose into. He then spent two seasons as a field crew leader for the United States Fish and Wildlife Service in Alaska, where he was privileged to work with some of the finest individuals one could know in the task of tracking Pacific salmon as far upstream as they could be found.

Seeking to top the experience of flying around Alaska in a helicopter, Mr. Arnold married his college crush on October 16, 2010. After stints as a land manager, carpenter, woodworker, and poultry farmer, he entered graduate school at Appalachian State University as a research assistant for Dr. Susan L. Edwards. On the first day of classes in August of 2012, he was blessed with the birth of his son, Micah. He received his Master of Science in December 2013.

Mr. Arnold currently lives in Valle Crucis, North Carolina with his wife, Joanna, and son, Micah. He is grateful to be surrounded by such an outstanding community and vibrant natural beauty, both of which have inspired many adventures and will not soon be forgotten.